

STUDIES ON AUTOIMMUNITY IN RELATION TO MEDICINE

William James Irvine



VOLUME 2

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CHAPTER IV

CHAPTER IVA CLINICAL AND IMMUNOLOGICAL STUDY OF IDIOPATHIC  
ADRENAL FAILURE, PREMATURE OVARIAN FAILURE AND  
IDIOPATHIC HYPOPARATHYROIDISM

"A Clinical and Immunological Study of  
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A CLINICAL AND IMMUNOLOGICAL STUDY OF  
ADRENAL INSUFFICIENCY

W. J. Irvine

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**A clinical and immunological study of adrenal insufficiency.** By W. J. IRVINE.

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The patients included in this study were subdivided into those in whom the adrenal insufficiency was idiopathic (15 patients), tuberculous (7 patients) or secondary to hypopituitarism (11 patients). A tuberculous aetiology was established by the presence of adrenal calcification on X-ray of the abdomen.

Four of the patients in the idiopathic group had associated thyroid disease; two gave a history of thyrotoxicosis, one had hypothyroidism and one had a goitre that was probably of the Hashimoto type. The patient with the Hashimoto goitre also had rheumatoid arthritis and a family history of pernicious anaemia. A fifth patient had latent pernicious anaemia; another patient had a family history of hypothyroidism; two further patients had diabetes mellitus. This contrasts with the absence of these diseases in the patients with tuberculous adrenal insufficiency or in their relatives. One patient in the hypopituitary group had a family history of pernicious anaemia.

Complement-fixing antibodies to adrenal, stomach and thyroid were found in 8, 6 and 5 of the 15 patients with idiopathic adrenal insufficiency, and antibodies to thyroglobulin in 9 of these patients. Two patients had no detectable levels of any of these antibodies and two patients possessed all four types of antibody. In the tuberculous group only one patient had a low titre of antibody to thyroglobulin and two of the hypopituitary patients, including the patient with a family history of pernicious anaemia, had complement-fixing antibody to gastric mucosa. The fluorescent antibody technique verified the results obtained by the complement-fixation method; it localized the site of antigen-antibody reaction in the adrenal to the cytoplasm of the secretory cells of the adrenal cortex, in the thyroid to the cytoplasm of the thyroid secretory epithelium, and in the gastric mucosa to the cytoplasm of the parietal cells. Absorption procedures and control studies with liver tissue established the specificity of the separate antigen-antibody reactions for the individual tissues.

Augmented histamine tests and/or gastric biopsies were carried out on thirteen of the idiopathic group, six of the tuberculous group and eight of the hypopituitary patients. Achlorhydria or minimal acid secretion was found in all three groups, while other patients in each group had gastric acid values that were either within or approached the normal range. The presence of gastric antibodies was associated with a histamine-fast achlorhydria in seven out of the nine cases in which they were detected. The autoimmune nature of gastric antibody was demonstrated in the one patient in whom a sufficient number of parietal cells persisted. The degree of atrophic gastritis was considered to be more severe in the idiopathic group.

It is concluded that idiopathic adrenal insufficiency is associated with a fundamental disorder of immunological homeostasis or tolerance and that the occurrence of circulating antibodies is not simply the result of tissue damage. Chronic thyroiditis, pernicious anaemia and idiopathic adrenal insufficiency may be basically similar in their pathogenesis, only differing in the tissue that is principally affected.

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A clinical and immunological study of adrenocortical insufficiency  
(Addison's disease)

W. J. IRVINE, A. G. STEWART AND LAURA SCARTH

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## A CLINICAL AND IMMUNOLOGICAL STUDY OF ADRENOCORTICAL INSUFFICIENCY (ADDISON'S DISEASE)

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### SUMMARY

Fifty-one patients with adrenocortical insufficiency were subdivided into three groups according to the nature of their adrenal disease; twelve patients with idiopathic, twenty-three patients with probable idiopathic and sixteen patients with tuberculous adrenal insufficiency. The importance of objective confirmation of a clinical diagnosis of adrenal insufficiency is stressed and the difficulties of classification of many patients with adult onset adrenal insufficiency are discussed. Idiopathic and probable idiopathic adrenal insufficiency had a sex ratio that was predominantly female (2.5:1) with a mean age of onset of 33 years.

Antibodies to adrenal cortex were detected by the methods of immunofluorescence and complement fixation. They were detected in the serum of 80% (20:25) of the females with idiopathic or probable idiopathic adrenal insufficiency and in only 10% (1:10) of the males. The titre of the adrenal antibody was low ( $\leq 32$ ) as tested either by immunofluorescence or complement fixation. The serum of only one patient with tuberculous adrenal insufficiency reacted with adrenal tissue in the complement fixation test but the immunofluorescence method showed that this serum reacted with the vascular endothelium and not the secretory cells. No correlation was observed between the duration of the clinical illness and the presence, or absence, or titre of the adrenal antibody. Adrenal antibody was not detected in the sera of fifty-one control subjects matched for age and sex. Four of sixty-nine patients with lymphadenoid goitre, one out of ninety-three patients with diabetes mellitus and none of 230 patients with thyrotoxicosis, primary hypothyroidism or pernicious anaemia had antibody in the serum specific for adrenocortical secretory cells.

There is a clinical and immunological overlap between idiopathic adrenal

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insufficiency and other diseases associated with autoimmune phenomena—thyroid disease, atrophic gastritis and hypoparathyroidism.

It is concluded that idiopathic adrenal insufficiency belongs to a group of diseases that are characterized clinically by onset predominantly in females, by aggregation in the same group of patients, by familial tendency, by the presence of organ-specific antibodies in the serum and histologically by lymphocytic infiltration and atrophy. While only one tissue of the group may be predominantly affected, the other tissues are frequently the site of subclinical disease. There is no set order in which the different tissues are affected. Serological evidence of adrenalitis is rare in patients with thyroid disease or pernicious anaemia, but thyroiditis and gastritis are common in patients with adrenalitis.

Two autopsies on patients with idiopathic adrenal insufficiency are described. One case had the classical features of adrenal atrophy with gross reduction in size of the glands, islets of secretory epithelial cells and a moderate degree of lymphocytic infiltration. In the other case, the adrenal glands were only slightly less than normal in size, there were few adrenal epithelial cells and a dense lymphocytic infiltration and some fibrosis. The histology resembled that of the thyroid in Hashimoto goitre.

If the term 'autoimmune' adrenalitis is to be substituted for idiopathic adrenal insufficiency, it should be on the understanding that while autoimmune mechanisms may be of primary importance in the pathogenesis of adrenal disease this has yet to be proven.

## INTRODUCTION

Adrenal antibodies have been detected in the sera of a proportion of patients with adrenal insufficiency (Anderson *et al.*, 1957; Mead, 1962; Blizzard *et al.*, 1962). Irvine (1963a) in a preliminary communication reported that antibodies specific for adrenal occurred in the sera of some patients with idiopathic adrenal insufficiency but not in the sera of patients with adrenal insufficiency of tuberculous origin. The present paper presents a more detailed clinical and immunological assessment of patients with idiopathic and non-idiopathic adrenal insufficiency.

## PATIENTS STUDIED

Fifty-one patients with primary adrenocortical insufficiency (Addison's disease) were studied. Diagnostic criteria were reviewed using where possible adequate adrenal stimulation with adrenocorticotrophin (ACTH) or with  $\beta_{1-24}$  ('Synacthen', Ciba) by infusion as described by Landon *et al.* (1964). Any patient was excluded from the present study who did not have Addison's disease when reviewed, or where the diagnosis seemed in reasonable doubt. Thirty-three of the fifty-one patients studied have been seen personally and most of them have been supervised regularly over a period of years at the Endocrine Clinic, Royal Infirmary, Edinburgh. On clinical, biochemical and radiological assessment, and independent of the immunological findings reported in this paper, the patients were subdivided into three groups (Tables 1-4).



*Adrenal insufficiency*

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*(1) Idiopathic adrenal insufficiency*

In addition to the usual clinical and laboratory criteria of the disease, eleven of the twelve patients in this group were known to have no response to adequate adrenal stimulation by measurement of plasma cortisol (Mattingly, 1962) or urinary excretion of 17-hydroxycorticosteroids (17-OHCS). In the remaining patient, the clinical diagnosis was confirmed at autopsy (case 1 of post-mortem studies). None of these patients had any personal history of tuberculosis and none had radiological evidence of adrenal calcification.

*(2) Probable idiopathic adrenal insufficiency*

The diagnosis of primary adrenal insufficiency in the twenty-three patients in this group was based on the classical clinical features, substantiated in most instances by studies of urinary 17-OHCS excretion, ability to excrete a water load before and after cortisone, and by insulin sensitivity. While a number of patients in this group received ACTH, the dosage was either unknown or inadequate and it is not possible at the present time to be certain of the potency of the preparation used. None of the patients in this group were known to have had adequate adrenal stimulation studies. In none of these patients was there any evidence of adrenal calcification but in an occasional case (e.g. No. 18) the possibility of tuberculosis of the adrenal could not be entirely excluded.

*(3) Tuberculous adrenal insufficiency*

There were sixteen patients in this group, nine of whom had radiological evidence of adrenal calcification as illustrated in Fig. 1. In seven of these nine patients there was clinical and radiological evidence of gross tuberculous disease in other organs of the body. In one (No. 39) there was evidence of a minimal lung lesion and, in the remaining patient (No. 44), there was no evidence of past tuberculosis other than adrenal calcification.

In the other seven patients in this group there was no radiological evidence of adrenal calcification; six of these patients had evidence of gross tuberculous infection elsewhere in the body. The remaining patient (No. 46) had an acid-alcohol fast bacilluria with a small chest wall abscess, but mycobacterium tuberculosis was not cultured.

In Group I patient No. 6 had experienced intermittent bronchospasm for many years and a faint apical opacity had occasionally been reported in the chest X-ray. The Mantoux reaction was reported to be just positive at 1:1000 dilution, and tuberculosis had occurred in one relative. Although there was no past history of any pertinent illness it is possible that this patient had tuberculous adrenal insufficiency. Patient No. 18 in Group II also had a positive Mantoux reaction and a history of tuberculosis in a relative. Her sister was said to have had erythema nodosum shortly before the patient's original admission and the patient herself was noted to have a pleural friction rub at that time. Although no tubercle bacilli were isolated she received antituberculous chemotherapy, and now has no radiological evidence of tubercle.

The Wassermann reaction was negative in all patients with idiopathic or probable idiopathic adrenal insufficiency except in patients Nos. 8 and 26. Patient No. 8 was stated to have had 'syphilitic labyrinthitis' in 1937 at the age of 47 and had a positive Wassermann reaction in 1958 but at autopsy (case 1 of post-mortem studies) there was no evidence of syphilis. Patient No. 26 was reported to have a weakly positive Wassermann and Kahn test on one occasion at a routine medical examination but he had no symptoms or signs

suggestive of syphilis. He received a course of penicillin and all subsequent Wassermann and Kahn tests were negative.

The age and sex distribution of the patients studied is summarized in Table 1.

The clinical features and the criteria for classifying the patients into the respective sub-groups are summarized in Tables 2-4.

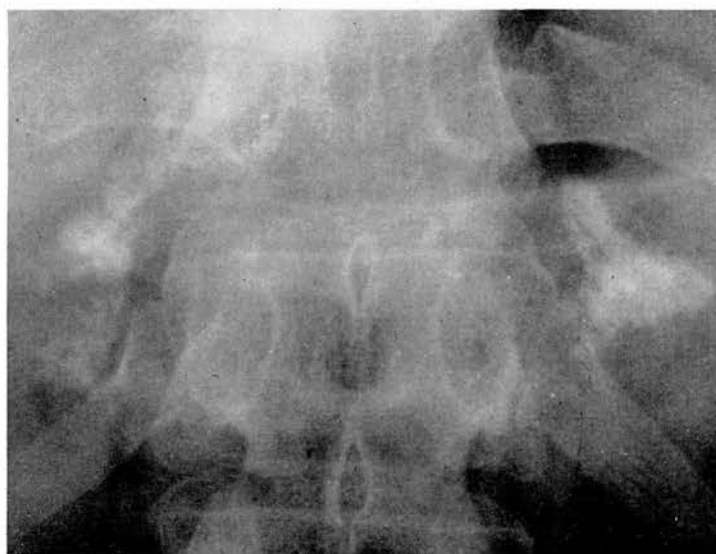


FIG. 1. Adrenal calcification on straight X-ray of abdomen.

TABLE 1. Adrenal insufficiency

Type	No. of patients	Age (years) at diagnosis of adrenal insufficiency		Age (years) at time of present study		Sex ratio Female:Male
		Range	Mean	Range	Mean	
Idiopathic	12	7-74	36	9-74	42	3.0:1.0
Probable idiopathic	23	15-63	33	19-66	43	2.3:1.0
Tuberculous	16	12-69	38	21-72	49	0.6:1.0

The sera of fifty-one blood donors, who were selected according to sex and age to match the fifty-one patients with adrenal insufficiency, and the sera of sixty-nine consecutive patients with lymphadenoid goitre, eighty-seven with primary hypothyroidism, eighty-nine with thyrotoxicosis, fifty-four with pernicious anaemia and ninety-three with diabetes mellitus were also studied.

The cord blood of two babies whose mothers had adrenal insufficiency were studied, and the serum was available from nine relatives of four patients with adrenal insufficiency.

## Adrenal insufficiency

TABLE 2. Clinical summary of patients with idiopathic adrenal insufficiency

Case No.	Name	Sex	Age	Age at onset	Associated disease (age at onset)	Family history	Chest X-ray	Adrenal calcification on X-ray	Mantoux or Heaf	Clinical details
1.	M.M.H.	F	41	38	—	—	Normal	None	—	Clinical features of Addison's disease including buccal pigment. Low urine 17-OHCS with response to ACTH 60 i.u. I.M.B.D. for 2 days.
2.	M.H.	F	17	12	Hypoparathyroidism (1½)	—	Normal	None	—	Clinical features: no rise in urine 17-OHCS with ACTH (25 i.u. by infusion). Skeletal anomalies. Intracranial calcification.
3.	A.N.	F	22	21	—	Thyroiditis, Diabetes mellitus, Rheumatoid arthritis	Normal	None	—	Clinical features including hyponatraemia. Low urine 17-OHCS and low plasma cortisol. No response to I.V. Synacthen.
4.	J.M.G.	F	52	50	—	—	Normal	None	Negative	Clinical features including buccal pigment. No response in urine 17-OHCS to ACTH 60 i.u. I.M.B.D. for 2 days.
5.	A.G.	F	38	37	—	Asthma	Normal	None	Negative	Clinical features. No response in urine 11-OHCS or in plasma cortisol to ACTH 40 i.u. I.M. daily for 2 days with I.V. Synacthen.
6.	C.S.	F	54	33	Myxoedema (44) Asthma (from teens) Urticaria	Tuberculosis	Very faint opacity left apex	None	Weak positive 1:1000	Clinical features only. No response in urine 17-OHCS to ACTH 40 i.u. I.M.B.D. for 2 days or in plasma cortisol to Synacthen infusion.
7.	E.A.	F	64	51	Thyroiditis (46)	—	Normal	None	Positive 1:1000	Clinical features only. No response to I.M. Synacthen 60 i.u. B.D. or to ACTH 40 i.u. I.M.B.D. for 3 days.
8.	D.M.	F	74	74	Pernicious anaemia (63); Syphilis (48)	—	(Post mortem report)	None	—	Clinical features including buccal pigment and hyponatraemia. Post-mortem series.
9.	J.S.	F	47	44	Diabetes mellitus (45)	—	Normal	None	—	Clinical features. No response in urine 17-OHCS to ACTH 100 i.u. I.M. daily for 3 days.
10.	B.H.	M	25	17	Diabetes mellitus (17)	Asthma Tuberculosis	Normal	None	Negative	Clinical features including buccal pigment and hyponatraemia. No response in plasma cortisol to Synacthen infusion with ACTH 40 i.u. I.M. once.
11.	J.F.	M	9	7	Schilder's disease (8)	Thyroiditis Diabetes mellitus Rheumatoid arthritis Addison's disease, Tuberculosis	Normal	None	—	Clinical features plus urine and plasma steroids. No response in urine 17-OHCS to two separate batches of ACTH.
12.	F.P.	M	59	49	'Latent' pernicious anaemia (55)	Pernicious anaemia	Normal	None	—	Clinical features: Kepler (±Cortisone), urine 17-oxosteroids, insulin sensitivity, Thorn positive twice. No response in urine 17-oxosteroids to ACTH 60 i.u. I.M. for 2 days.

i.u., International units; B.D., twice daily; I.M., intramuscularly.

TABLE 3. Clinical summary of patients with probable idiopathic adrenal insufficiency

Case No.	Name	Sex	Age	Age at onset	Associated disease (age at onset)	Family history	Chest X-ray	Adrenal calcification on X-ray	Mantoux or Heaf	Clinical details
13.	C.K.	F	45	27	Asthma (from late 30s) Thyrototoxicosis (27)	Diabetes mellitus, Pernicious anaemia, Asthma	Normal	None	—	Clinical features including buccal pigment Doubtful water load, and insulin sensitivity. No response in urine 17-oxosteroids to ACTH 25 i.u. I.V. on 2 days.
14.	C.C.	F	33	23	Diabetes mellitus (31)	Diabetes mellitus Thyrototoxicosis, Tuberculosis, Asthma	Normal	None	—	Clinical features only.
15.	W.C.	F	24	24	Hypoparathyroidism (12) Moniliasis	Rheumatoid arthritis	Normal	None	—	Clinical features including hyponatraemia, plus low urine steroids, and steroid recovery from 'crises'; bilateral ptosis; vitreous opacities; calcified basal ganglia.
16.	D.S.	F	22	21	Childhood eczema and asthma	—	Normal	None	Negative	Acutely ill at diagnosis. Clinical features. Kepler test, steroid resuscitation. Transient apical consolidation cleared immediately with streptomycin. Subsequent Heaf negative.
17.	M.T.	F	33	31	Diabetes mellitus (25)	—	Normal	None	—	Clinical features including buccal pigment and hyponatraemia. Kepler corrected with cortisone. Doubtful plasma cortisol figures but no response in these to ACTH 25 i.u. by infusion.
18.	G.J.	F	19	15	—	Tuberculosis	Normal	None	Positive	Clinical features with buccal pigment 'crisis' during ACTH test. 'Cardiomegaly'. No tubercle cultured.
19.	G.C.	F	47	45	Thyrototoxicosis (47)	Diabetes mellitus thyroid surgery	—	None	—	Clinical features. No plasma cortisol response to I.M. Synacthen.
20.	M.L.	F	20	15	Hypoparathyroidism (16) Alopecia totalis Moniliasis Pernicious anaemia (21)	'Coeliac syndrome' with early death in brother Moniliasis	—	None	—	Clinical features including collapse, low blood sugar, steroid resuscitation. Low urine 17-oxo-steroids and 17-OHCS.
21.	A.G.	F	48	36	—	—	Normal	None	Positive	Clinical features only.



## Adrenal insufficiency

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22.	C.S.	F	59	45	Diabetes (58)	Diabetes mellitus Peptic ulcer	Small calcified focus	None	—	Clinical features. Low urine 17 oxosteroids. Positive Kepler and Thorn tests.
23.	J.F.	F	64	49	—	—	Normal	None	—	Clinical features including hyponatraemia and buccal pigment.
24.	M.S.	F	42	24	—	Diabetes mellitus Rheumatoid arthritis Hypothyroidism Carcinoma of stomach	Normal	None	—	Clinical features including buccal pigment. No plasma cortisol response to I.M. Synacthen. Acute febrile illness aged 25 years for which she received antituberculous chemotherapy.
25.	R.S.	M	23	21	Diabetes (21)	—	Normal	None	—	Clinical features including hyponatraemia.
26.	J.S.	M	65	51	Vitiligo	Thyroiditis Asthma	Small calcified focus in apex of lower lobe	None	—	Clinical features including hyponatraemia. Weak positive W.R. age 53 years.
27.	M.McG.	F	62	46	—	—	Small calcified focus in right mid-zone	None	—	Clinical features. No plasma cortisol response to I.M. Synacthen.
28.	G.S.	M	37	17	? Hypothyroidism	—	Normal	None	—	Clinical features including hyponatraemia. No plasma cortisol response to I.M. Synacthen.
29.	A.D.	F	53	34	—	Rheumatoid arthritis	Normal	None	—	Clinical features including buccal pigment.
30.	M.McK.	F	62	51	Rheumatoid arthritis (57)	Pericious anaemia	Normal	None	—	Clinical features with hyponatraemia and buccal pigment. Low urine 17 oxosteroid. Failure to excrete water load.
31.	J.M.	M	66	63	—	—	Normal	None	—	Clinical features with hyponatraemia.
32.	A.A.	F	22	16	Moniliasis	Goitre: Asthma	Normal	None	Negative 1:1000	Clinical features only. No response in plasma cortisol to Synacthen infusion (age 22). Normal thyroid and parathyroid function studies.
33.	D.W.	M	32	15	—	—	Normal	None	—	Clinical features with hyponatraemia.
34.	D.T.	M	48	41	Diabetes mellitus (30)	—	Normal	None	—	Clinical features with hyponatraemia. Low urine 17 ketogenic steroids. Failure to excrete water load. Diabetic retinopathy and neuro- pathy.
35.	G.F.	M	53	44	—	Diabetes mellitus	Calcified focus	None	—	Clinical features with buccal pigment and hyponatraemia. Low urine 17 oxosteroids.

TABLE 4. Clinical summary of patients with tuberculous adrenal insufficiency

Case No.	Name	Sex	Age	Age at onset	Associated disease	Family history	Chest X-ray	Adrenal calcification on X-ray	Mantoux or Heat	Clinical details
36.	M.S.	F	49	26	Radiographic vertebral fusion	Thyrototoxicosis	Old apical lesions	Present	—	Clinical features only.
37.	J.N.	F	45	35	Proved renal tubercle hip, spine, peritoneum	Diabetes mellitus Tuberculosis	—	Present	—	Clinical features. No response in urine 17-oxosteroids or electrolyte ratios to ACTH. 25 i.u. by infusion. Mitral stenosis.
38.	R.G.	M	46	30	Pleural effusions	'Bright's disease'	Pleural and apical shadow	Present	—	Clinical features. No plasma cortisol response to I.M. Synacthen.
39.	H.S.	F	30	17	? Peritonitis	Thyrototoxicosis	Minimal lung lesion	Present	—	Clinical features. Low urine 17-oxosteroids. Poor water excretion corrected with cortisone.
40.	G.McD.	M	44	34	T.B. spine	—	Bilateral apical lesions	Present	—	Clinical features with hyponatraemia. Positive Thorn test. Alcoholic.
41.	M.K.	M	21	18	—	Tuberculosis	Lower lobe calcifications	Present	Positive	Clinical features of acute onset. No response in urine 17-OHCS to ACTH.
42.	A.H.	F	55	43	Proved T.R. of elbow and femur	Tuberculosis	Normal	Present	—	Clinical features including hyponatraemia.
43.	S.C.	F	72	68	'Pulmonary' tubercle	Diabetes mellitus	Bilateral apical lesions	Present	—	Clinical features with no response in 17-OHCS excretion on ACTH 60 i.u. B.D.I.M. for 2 days. Duodenal ulcer. Carcinoma uterus.
44.	G.C.	M	41	24	Partial small bowel obstruction	—	Normal	Present	—	Clinical features only. Duodenal ulcer.
45.	A.S.	M	52	45	Pleurisy	—	Pericardial calcification	Doubtful	—	Clinical features with buccal pigment and hyponatraemia.
46.	J.W.	M	46	38	Abscess chest wall Acid-alcohol fast bacilluria	—	Normal	None	—	Clinical features with hyponatraemia. Low urine 17-oxo and 17-OHCS.
47.	J.H.	M	67	52	Alopecia totalis Pleural effusions 'Acid-fast' sputum	—	Pleural shadow Upper zone opacity	None	—	Clinical features with hypoglycaemia and insulin sensitivity. No plasma cortisol response to I.M. Synacthen.
48.	C.H.	M	39	28	Proved lung and renal tuberculosis	Diabetes mellitus Asthma	Bilateral pulmonary lesions	None	—	Clinical features. No response in plasma cortisol to I.M. Synacthen.
49.	R.F.	F	70	68	T.B. abscess of spine	—	—	None	Positive	Clinical features with buccal pigment and no response in plasma cortisol to Synacthen infusion or in urine 17-OHCS to ACTH 40 i.u. I.M.B.D. for 3 days.
50.	B.F.	M	29	12	T.B. lung, neck glands, testis	—	—	None	—	Clinical features only.
51.	J.D.	M	72	69	T.B. neck glands, gut, kidney	—	Apical opacities	None	Positive 1:10000	Clinical features only.

## Adrenal insufficiency

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The patients with adrenal insufficiency were repeatedly asked about the history of relevant diseases in other members of their families, but in only a few instances were the relatives seen and examined by ourselves.

### METHODS

#### *Fluorescent antibody technique*

The indirect fluorescent antibody technique (Coons & Kaplan, 1950) was used. Air-dried, unfixed sections of human adrenal, human gastric body mucosa, human thyrotoxic gland and of rat liver were cut at  $4\mu$  using a Pearce cryostat at  $-20^{\circ}\text{C}$ .

Adrenal tissue was obtained from two patients. Adrenal No. 1 was from a boy (J.W. aged 11 years) who underwent bilateral adrenalectomy for Cushing's disease. The diagnosis of Cushing's disease was based on the clinical features and substantiated by a raised urinary excretion of 17-OHCS and raised plasma levels of 11-OHCS with no diurnal variation. As described in some 10% of patients with this condition (Williams, 1962) the adrenal glands were of normal size, weight (4.5 and 5.0 g) and histology. The blood group was A Rh positive. Adrenal No. 2 was from a female aged 55 years who underwent bilateral adrenalectomy for carcinomatosis having previously been treated with an anabolic steroid. She had received 100 mg cortisone the day before operation and 75 mg cortisone on the day of operation. The blood group was O Rh positive.

As soon as each adrenal gland had been excised, rectangular blocks of tissue of approximately 2–3 mm thickness were cut at right angles to the surface of the gland, placed in small glass vials and rapidly frozen to  $-70^{\circ}\text{C}$  in acetone–solid  $\text{CO}_2$  mixture. The blocks were stored in  $-20^{\circ}\text{C}$  until required for sectioning. They were mounted in the cryostat so that they could be cut in cross-section. In this way it was ensured that each section of adrenal contained capsule, zona glomerulosa, zona fasciculata, zona reticularis. Most but not all of the sections contained medulla.

Mucosa from the body of human stomach was obtained from a patient undergoing gastrectomy and human thyroid tissue was obtained at partial thyroidectomy for thyrotoxic goitre after preparation with anti-thyroid drugs and potassium iodide. A biopsy of human testicular tissue was obtained at prostatectomy and vasectomy; the histology of the testicular tissue was normal.

The sections of the various tissues may be mounted on individual glass slides or grouped conveniently on the same slide. The sections were air-dried by fan for 1 hr, stored at  $4^{\circ}\text{C}$  and used within 3 days. During storage the sections were protected in polythene bags from condensation and prior to use the sections were allowed to come to room temperature before being removed from the polythene bag.

A number of different fluorescein–protein conjugates were studied and are described under two headings. Fluorescein was used throughout in the form of fluorescein isothiocyanate (FITC).

(1) *Various commercial preparations using polyvalent antisera.* The most satisfactory of the commercial preparations examined was prepared in the following manner. Normal human globulin was injected intramuscularly with adjuvant into horse at intervals to maximum titre. The horse serum was then fractionated with half-saturated ammonium sulphate. After removal of all sulphate the globulin fraction was conjugated with FITC absorbed

with liver powder and dialysed free of fluorescein. The antibody titre of the serum was 1:480 and the molar fluorescein-protein ratio was 8.8  $\mu\text{g}/\text{mg}$  and the fluorescein to protein absorption ratio was 0.87.

(2) *Preparations using monovalent antisera.* Anti-human IgG, anti-human IgM and anti-human  $\beta_{1C}$  (each prepared in horse) were obtained from the Central Blood Transfusion Laboratory, Netherlands Red Cross, Amsterdam. The specificity of the IgG and IgM anti-sera was verified by immunoelectrophoresis. For the purpose of conjugation the globulin fraction of each antiserum was diluted to approximately 10 mg/ml with carbonate-bicarbonate buffer at pH 9.0. Chromatographically pure Isomer I FITC (Baltimore Biological Laboratories) was used in a concentration of 20 mg/100 mg protein. The conjugation was done at 4°C and the time of conjugation was 22 hr. Free fluorescein was removed by filtration through Sephadex G-50 followed by overnight dialysis in phosphate buffered saline at 4°C. The conjugates were used in a dilution that gave optimum specific fluorescein staining with minimum non-specific staining. The fluorescein-protein absorption ratios of the conjugates using anti-IgG, anti-IgM and anti- $\beta_{1C}$  were 0.52, 1.1 and 0.83 respectively.

The sera were stored at -20°C. Undiluted serum after warming to room temperature was applied to each section for 20 min at the same temperature. The sections were then washed in phosphate buffer (pH 7.2) for 30 min with continual gentle agitation, the buffer being changed once during this procedure. The fluorescein-protein conjugate was applied for 20 min. When the protein moiety of the conjugate was anti-human  $\beta_{1C}$ , the sections were treated with fresh normal human serum for 20 min following the application of the test serum. After application of the conjugate, all sections were given a final wash in phosphate buffer pH 7.2 for 1 hr with continuous agitation. In some instances the sections were then counterstained with papain conjugated with rhodamine (Alexander & Potter, 1963). At the end of the staining procedure the sections were mounted in 10% glycerol in saline and examined under ultraviolet light within 2 hr using a Zeiss Universal microscope fitted with a super-pressure mercury lamp HBO.200 W, exciter filters BG 38 and BG 12, direct light or dark ground condenser and 530 m $\mu$  barrier filter. After preliminary comparisons of the different conjugates, anti-IgG and anti-IgM conjugates were used separately in testing sera for antibody to human adrenal, to epithelial cytoplasm of human thyrotoxic tissue, to parietal cells in human gastric mucosa and for antinuclear factors using rat liver. Anti- $\beta_{1C}$  conjugate was also used for detecting antibody to adrenal. All the sera from patients with adrenal insufficiency were tested against sections of human adrenal gland No. 1 and adrenal gland No. 2. All other sera were tested against adrenal gland No. 1. Test sera were titrated for adrenocortical antibody using sections of adrenal No. 1, anti-IgG conjugate and doubling dilutions of sera. The intensity of staining was assessed subjectively and recorded as ++, +,  $\pm$  or negative.

#### *Complement fixation tests*

All sera were tested for complement fixing antibody against a saline extract of adrenal gland No. 1 using the Takatsy microtitre technique (Irvine, 1966b) after initial chess-board titration of adrenal antigen. Two M.H.D. of complement were used. The test was read visually. Fifty per cent haemolysis with a serum dilution of 1:4 or greater was taken as a positive result. The sera were inactivated at 56°C for  $\frac{1}{2}$  hr and were initially screened in doubling dilutions from 1:2 to 1:32 and further titrations were done subsequently if



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required to define the end point. A control was included for each serum to test for anti-complementary activity.

All sera were also tested for complement fixing antibody to saline extracts of human thyrotoxic tissue, mucosa of the body of pig stomach and rat liver. Both the manual microtitre method and the AutoAnalyzer method were used for this purpose (Irvine, 1966b; Irvine & Marwick, 1967).

The sheep red cells in Alsever solution, rabbit haemolytic serum and preserved guinea-pig complement were obtained from Stayne Laboratories, High Wycombe, Buckinghamshire.

*Absorption procedures*

The specificity of the reactions to adrenal tissue observed by the immunofluorescent and complement fixation techniques was studied by absorption of the antibody by saline extracts of individual tissues (the human thyroid, pig stomach, rat liver and human adrenal No. 1). The highest concentration of the tissue extract that was not anti-complementary was used. Three parts of tissue extract to one part undiluted test serum were incubated with continual mixing at room temperature for 3 hr and then centrifuged at 1750 g for 10 min. Samples before and after absorption were then titrated by the manual microtitre complement fixation test and the indirect immunofluorescent technique using anti-IgG conjugated with FITC.

*Other serological procedures*

Sera were screened and titrated for antibody to thyroglobulin by the tanned cell haemagglutination method (Fulthorpe *et al.*, 1961) using reagents supplied by Burroughs Wellcome, Beckenham, Kent. The sera were also screened and titrated for antibody to intrinsic factor using the radioisotope technique with charcoal absorption (Irvine, 1966a). The electrophoretic pattern of the serum using cellulose acetate and the erythrocyte sedimentation rate were determined in the patients with idiopathic adrenal insufficiency.

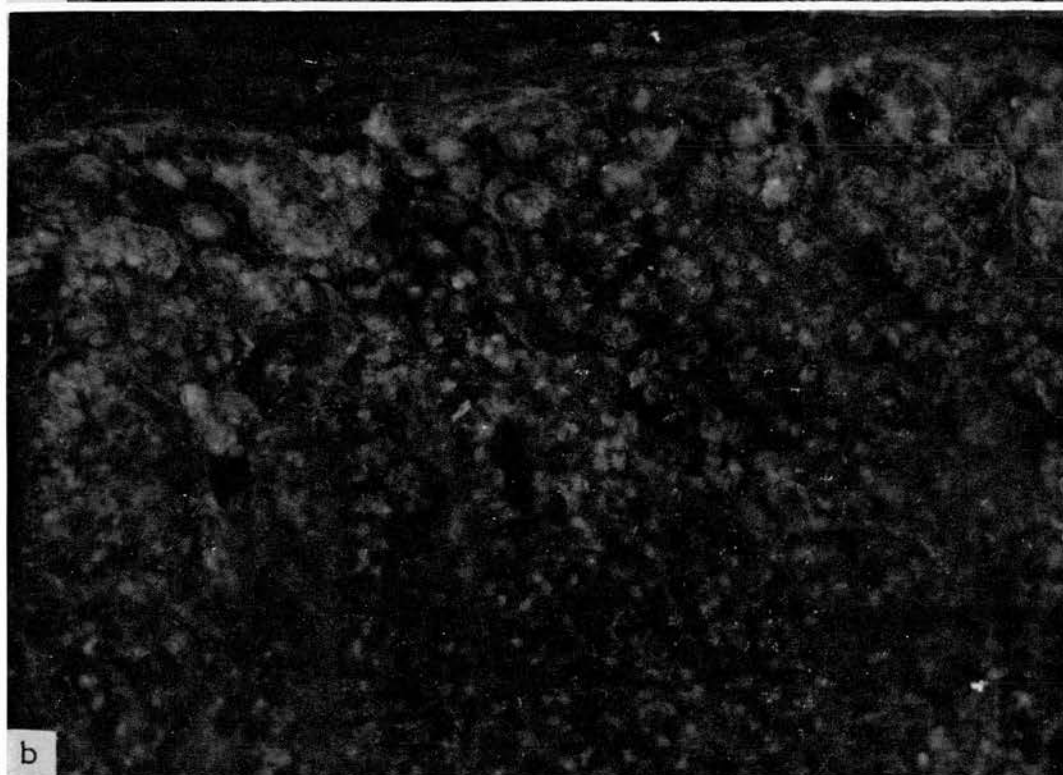
Wassermann and Kahn tests were done and the ABO and Rh blood groups were determined in all patients with adrenal insufficiency.

*Gastric, thyroid, parathyroid and pancreatic function tests*

Tests of gastric function were done on a proportion of the patients with adrenal insufficiency using the augmented histamine test (Kay, 1953). The dose of histamine acid phosphate was 0.04 mg/kg body weight and was given subcutaneously and preceded by an intramuscular injection of 50–100 mg mepyramine maleate  $\frac{1}{2}$  hr before the injection of histamine. The details of the technique of aspirating gastric juice were as described by Makhlof, McManus & Card (1964). The sample of gastric juice was estimated for acid content using an automatic titrator (Radiometer, Copenhagen) to pH 7.0. The content of intrinsic factor in the gastric juice was measured by radio immunoassay (Irvine, 1966a). Schilling tests of vitamin B<sub>12</sub> absorption (Schilling, 1953) and the level of vitamin B<sub>12</sub> in the serum was estimated by the microbiological method using *Lactobacillus leichmanii* as test organism and with the addition of cyanide to the serum (Girdwood, 1960).

Gastric biopsy using a Crosby capsule (Crosby & Kugler, 1957) was done on eighteen of the patients with adrenal insufficiency.

Thyroid function was assessed by estimation of the serum protein bound iodine (PBI) level using the automated procedure (Crowley & Jensen, 1965). When disorder of thyroid



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function was suspected clinically or indicated by an abnormal PBI, further studies were done using standard radioisotope techniques.

## RESULTS

*Detection and characterization of adrenocortical antibody*

The detection of antibody reactive with the secretory cells of the adrenal cortex is illustrated in Figs. 2 and 3, using sera from two patients with idiopathic adrenal insufficiency and from two control subjects. In each instance the fluorescent staining of the adrenocortical secretory cells was given by the anti-IgG or anti- $\beta_{1C}$  conjugates but not by the anti-IgM or anti-IgA conjugates. This was true of all sera tested for adrenal antibody. The intensity of the fluorescent staining was maximal in the zona glomerulosa. Specific staining of the zona fasciculata and zona reticularis was also observed but this was less marked than that of the zona glomerulosa. The fluorescent staining was confined to the cytoplasm of the adrenocortical secretory cells, the nuclei not being stained unless antinuclear factors were also present. The staining was finely granular and fairly evenly distributed throughout the cytoplasm of the cells of the zona glomerulosa. In the zona fasciculata the staining was particularly pronounced in the region of the cell membranes and surrounding the secretory globules within the cells. In the zona reticularis the pattern of immunofluorescence was again more even but still disrupted by the presence of secretory droplets that were autofluorescent. Fourteen of the total sera gave clear immunofluorescent staining of what appeared to be the vascular endothelium in the adrenal cortex and the adrenal medulla (Fig. 5). The staining of the vascular endothelium was given only with anti-IgG and anti- $\beta_{1C}$  conjugates and not with anti-IgM or anti-IgA conjugate. Only one serum out of the total of 492 sera showed immunofluorescent staining of the cells of the adrenal medulla (Fig. 6).

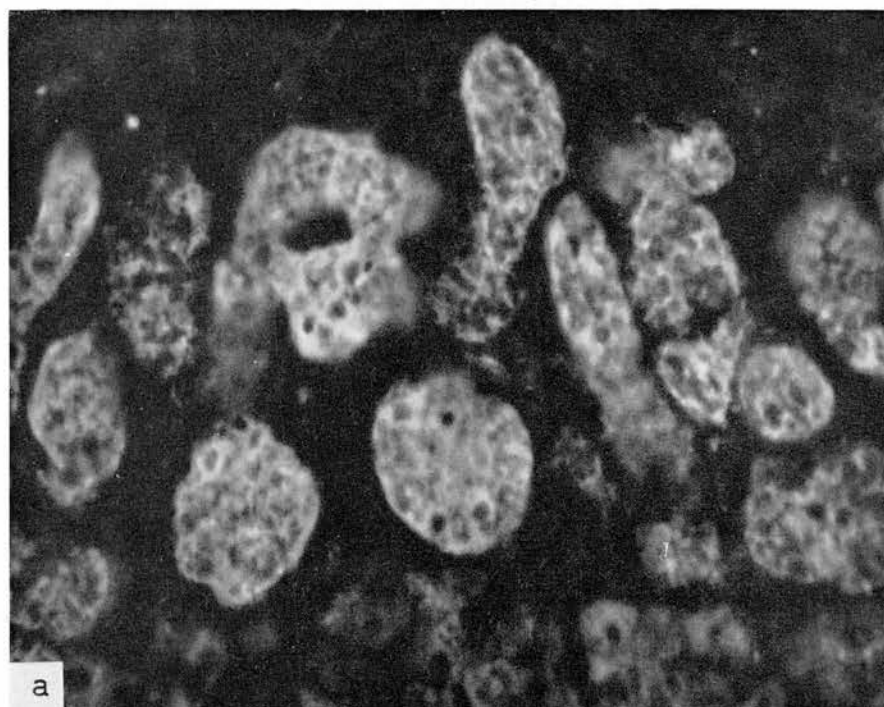
Sections of adrenal gland No. 2 gave the same results for adrenocortical staining as sections of adrenal gland No. 1 with the undiluted sera of the patients with adrenal insufficiency and using anti-IgG or anti- $\beta_{1C}$  conjugates. The immunofluorescent staining with the Cushing adrenal (adrenal gland No. 1) was considered to be better than with the adrenal gland removed from the patient with carcinomatosis (adrenal gland No. 2). Adrenal gland No. 2 did not show the staining of vascular endothelium observed with sections of adrenal gland No. 1.

In interpreting the immunofluorescence tests using adrenal tissue it is of particular importance to use thin sections of fresh tissue and that the section particularly in the region of the capsule and zona glomerulosa is complete and unbroken. Adrenal tissue is particularly liable to show background staining and every care must be taken in preparation of the conjugate used to demonstrate the interaction of the antibody in the test serum with the

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FIG. 2. (a) Indirect fluorescent antibody technique using an unfixed frozen section of an adrenal gland from a patient with Cushing's disease. The serum was used in a dilution of 1:4 and was from a patient with idiopathic adrenal insufficiency (No. 1). The antigen-antibody reaction was detected using horse anti-human immunoglobulin IgG conjugated with fluorescein isothiocyanate. Note that the intensity of the staining is greatest in the zona glomerulosa. (b) The same as (a) but using normal serum diluted 1:4 instead of serum from a patient with Addison's disease. Direct ultraviolet light,  $\times 250$ .







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adrenal antigen. The commercial preparation obtained by conjugating horse anti-human globulin serum with FITC did not give satisfactory results with adrenal tissue, although the same preparation may be adequate for detecting other types of antigen-antibody reaction.

Clear immunofluorescent staining of the adrenocortical cells was achieved using monovalent anti-human immunoglobulin (IgG) conjugated with FITC. The clarity of the result increases with the thinness of the section of adrenal tissue. Only in one instance was there difficulty in distinguishing a positive reaction from a negative reaction and the result had to be labelled as  $\pm$ . The fluorescent staining given by a particular serum was highly reproducible. Greater contrast between weak fluorescent staining and the low grade background staining was achieved with dark ground illumination. Counter staining the section with

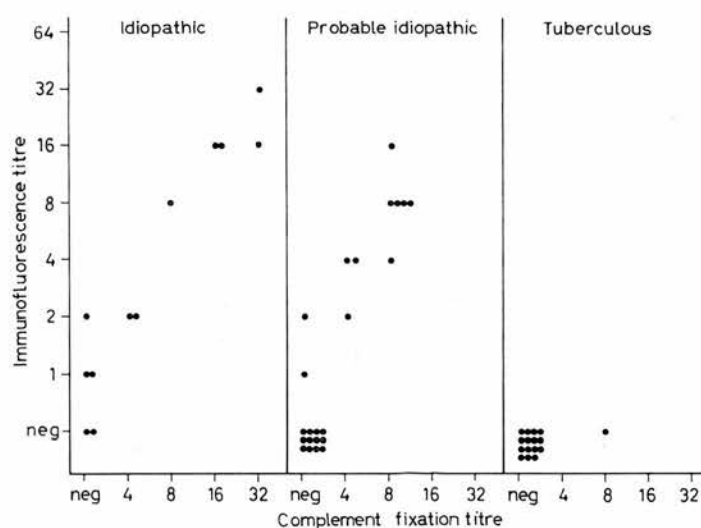
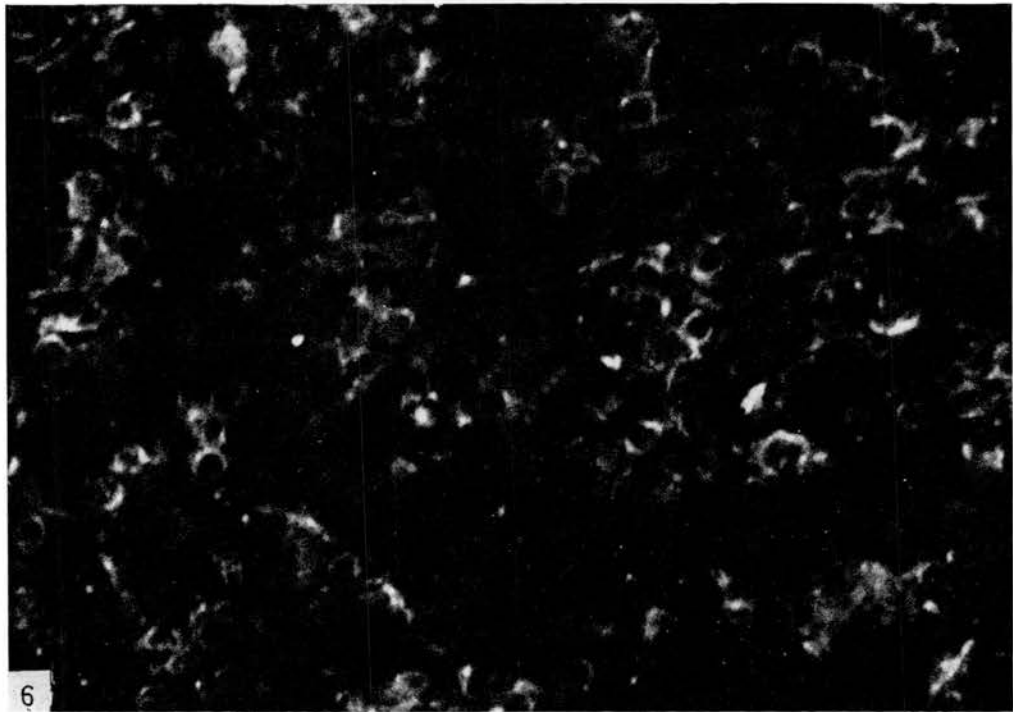


FIG. 4. The titration of adrenal antibody in the sera of patients with primary adrenocortical insufficiency using the methods of indirect immunofluorescence and complement fixation. The immunofluorescent staining was in the secretory cells of the adrenal cortex using anti-IgG conjugate. The serum of the patient in the tuberculous group that gave a positive complement fixation test with a saline extract of adrenal stained the capillary and sinusoidal endothelium in the indirect immunofluorescent test using anti- $\beta_{1C}$  conjugate, but did not stain the adrenocortical secretory cells.

papain conjugated with rhodamine was effective in removing the small amount of non-specific fluorescein staining encountered with the anti-IgG conjugate. Provided the papain-rhodamine was not used to excess, its use enhanced the clarity of the results. With adrenal sections of 1–2  $\mu$  thickness, undiluted test sera and anti- $\beta_{1C}$  conjugate there was no non-specific staining. Using optics as described above, the adrenal secretory cells (particularly the secretory globules in the zona fasciculata) show a pale brownish red autofluorescence.

FIG. 3. (a) Positive immunofluorescence in the secretory cells of the zona glomerulosa of adrenal cortex in the indirect fluorescent antibody technique using the serum from a patient (No. 2) with idiopathic adrenal insufficiency and anti IgG—F.I.T.C. conjugate. (b) Negative control. Dark ground ultraviolet light,  $\times 450$ .



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The presence of adrenal specific antibodies was confirmed using the method of complement fixation. As shown in Fig. 4 there is a good correlation between the titre for adrenal antibody as detected in the indirect immunofluorescent technique and the complement fixation test (using 2 M.H.D. of complement in the manual microtitre method) in the three groups of sera from patients with primary adrenal insufficiency. The maximum titre observed by either procedure was 1:32. None of the sera were anti-complementary and none gave a positive complement fixation test with rat liver. The one serum in the tuberculous group in Fig. 4 that gave a positive complement fixation test with adrenal extract No. 1 gave a negative immunofluorescent reaction with adrenocortical secretory cells but reacted strongly with the capillary and sinusoidal endothelium of that adrenal as illustrated in Fig. 5. The blood group of this patient was group O Rh positive. This serum did not react with rat liver, human thyrotoxic tissue, pig gastric mucosa nor did it contain anti-nuclear factors or appear to be anti-complementary.

Absorption studies showed that the complement fixing adrenocortical antibody and the immunofluorescent staining of adrenocortical secretory cells could be selectively absorbed with a saline extract of human adrenal but not with similar extracts of human thyroid or of pig gastric mucosa, even although these thyroid and gastric extracts were capable of absorbing thyroid and gastric antibodies, respectively.

It has not yet been possible to establish the autologous nature of the adrenocortical antibody. There was no correlation found between the blood group (ABO and Rh) of the test sera and that of the donor of the adrenal tissue with respect to the immunofluorescent staining of the adrenocortical secretory cells.

The staining of the vascular endothelium in adrenal gland No. 1 (blood group A Rh positive) shown by a few of the sera studied in the immunofluorescent test was not seen with sections of adrenal gland No. 2 (blood group O Rh positive) nor with thyroid tissue from another patient or with rat liver or kidney sections. These sera gave negative complement fixation tests with saline extracts of thyroid, stomach and liver and they did not contain antinuclear factors.

The concentration of  $\gamma$ -globulin in the serum of the patients with idiopathic adrenal insufficiency ranged from 0.6 to 1.4 g/100 ml and the average erythrocyte sedimentation rate was 15 mm in the first hour (Westergren). In patients Nos. 3, 9, 15 and 18 the average in each individual was between 20–39 mm. In these four patients the adrenocortical insufficiency was of recent onset but there was no obvious correlation with antibody titres or plasma electrophoretic pattern. In patient No. 15 the erythrocyte sedimentation rate was mildly but persistently elevated from the onset of hypoparathyroidism to the development of adrenal failure.

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FIG. 5. Staining of capillary and sinusoidal endothelium in an unfixed section of human adrenal gland No. 1 (Group A Rh positive) with the serum of patient No. 45, human complement and anti- $\beta_{1C}$ -FITC conjugate. The adrenocortical secretory cells are not stained. Direct ultraviolet light,  $\times 125$ .

FIG. 6. Staining of cells in the adrenal medulla observed with one serum (from a patient with lymphadenoid goitre) out of the 492 sera studied. The staining of cells is in the form of a ring surrounding the nucleus. Neither the adrenocortical cells nor the interstitial cells were stained. Anti- $\beta_{1C}$  conjugate. Dark ground ultraviolet light,  $\times 530$ .

TABLE 5. Serological findings in patients with idiopathic adrenal insufficiency

Case No.	Adrenal cortex		Thyroid			Gastric			Liver	ANF
	Fl.	C.F. (titre)	T.C.H.	Fl.	C.F.	Fl.	C.F.	I.F.		
1	++	32	5	+	4	+	neg	neg	neg	neg
2	++	32	neg	neg	neg	++	8	20	neg	neg
3	++	16	neg	neg	neg	neg	neg	neg	neg	neg
4	++	16	neg	+	neg	neg	neg	neg	neg	neg
5	++	8	neg	++	64	++	64	neg	neg	neg
6	+	4	2500	++	16	++	8	neg	neg	neg
7	+	4	neg	neg	neg	++	32	neg	neg	neg
8	+	neg	neg	++	32	neg	neg	39	neg	neg
9	+	neg	neg	neg	neg	neg	neg	neg	neg	neg
10	+	neg	neg	+	neg	neg	neg	neg	neg	neg
11	±	neg	neg	neg	neg	neg	neg	neg	neg	neg
12	neg	neg	neg	+	8	++	8	6	neg	neg

Fl., Immunofluorescence; C.F., complement fixation; T.C.H., tanned cell haemagglutination; I.F., intrinsic factor; ANF, antinuclear factors.

TABLE 6. Serological findings in patients with probable idiopathic adrenal insufficiency

Case No.	Adrenal cortex		Thyroid			Gastric			Liver	ANF
	Fl.	C.F. (titre)	T.C.H.	Fl.	C.F.	Fl.	C.F.	I.F.		
13	++	8	neg	neg	neg	neg	neg	neg	neg	neg
14	++	8	2500	+	16	++	128	neg	neg	neg
15	++	8	25	neg	neg	+	8	27	≥ 32	IgM
16	++	8	neg	+	8	neg	neg	neg	neg	neg
17	++	8	neg	neg	neg	neg	neg	neg	neg	neg
18	++	4	neg	—	32	neg	neg	neg	neg	neg
19	++	4	neg	++	32	++	16	neg	neg	neg
20	++	4	250	++	16	neg	neg	880	neg	neg
21	++	4	neg	neg	neg	neg	neg	neg	neg	neg
22	++	neg	neg	neg	neg	neg	neg	neg	neg	neg
23	+	neg	neg	neg	neg	++	32	8	neg	neg
24	neg	neg	neg	+	16	++	16	9	neg	neg
25	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
26	neg	neg	neg	neg	neg	++	8	neg	neg	neg
27	neg	neg	neg	++	32	++	4	neg	neg	neg
28	neg	neg	25	+	neg	+	4	neg	neg	neg
29	neg	neg	neg	+	8	neg	neg	neg	neg	neg
30-35	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

For abbreviations see Table 5.



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*Incidence of adrenocortical antibody*

The occurrence of adrenocortical antibody in the fifty-one patients with adrenal insufficiency and in control subjects is shown in Tables 5–8. The incidence of adrenocortical antibody according to the sex of the patients with adrenal insufficiency is summarized in Table 9. Ten out of the twelve patients in the idiopathic group had detectable amounts of adrenal antibody in the serum by the immunofluorescent method. The serum of one other patient in

TABLE 7. Serological findings in patients with tuberculous adrenal insufficiency

Case No.	Adrenal cortex		Thyroid			Gastric			Liver	ANF
	FL.	C.F.	T.C.H.	FL.	C.F.	FL.	C.F.	I.F.		
36	neg	neg	25	++	neg	neg	neg	neg	neg	neg
37	neg	neg	neg	+	16	neg	neg	neg	neg	neg
38	neg	neg	neg	neg	neg	++	4	neg	neg	neg
39–44	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
45	neg	8	neg	neg	neg	neg	neg	neg	neg	neg
46–51	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

For abbreviations see Table 5.

TABLE 8. Incidence of adrenal antibodies

	No. of patients	Antibody positive		
		Fluorescence		Complement fixation
		Secretory cells	Interstitial*	
<i>Adrenal insufficiency</i>				
Idiopathic	12	10	—	7
Probable idiopathic	23	11	—	9
Tuberculous	16	—	1	1*
<i>Controls (matched for age and sex)</i>	51	—	—	—
<i>Thyrotoxicosis</i>	89	—	1	1*
<i>Lymphadenoid goitre</i>	69	2	2	2
<i>Primary atrophic hypothyroidism</i>	87	—	7	2*
<i>Pernicious anaemia</i>	54	—	—	—
<i>Diabetes mellitus</i>	93	4	3	1

\* The antigen in this reaction was localized to capillary and sinusoidal endothelium and not to adrenal secretory cells, using anti- $\beta_{1C}$  conjugate in the indirect immunofluorescent technique.

the idiopathic group gave equivocal ( $\pm$ ) staining of adrenal tissue with anti-IgG conjugate but was negative with anti- $\beta_{1C}$  conjugate; the remaining serum in this group was negative. No other patient with adrenal insufficiency nor any of the control patients gave doubtful reactions. Antibody to the secretory cells of the adrenal cortex was detected in the sera of eleven out of the twenty-three patients with probable idiopathic adrenal insufficiency but

in none of the sixteen patients with tuberculous adrenal insufficiency using the fluorescent method. The complement fixation test (using the serum diluted 1:4) was, as expected, less sensitive as a screening procedure than the immunofluorescence test in which the serum was used undiluted.

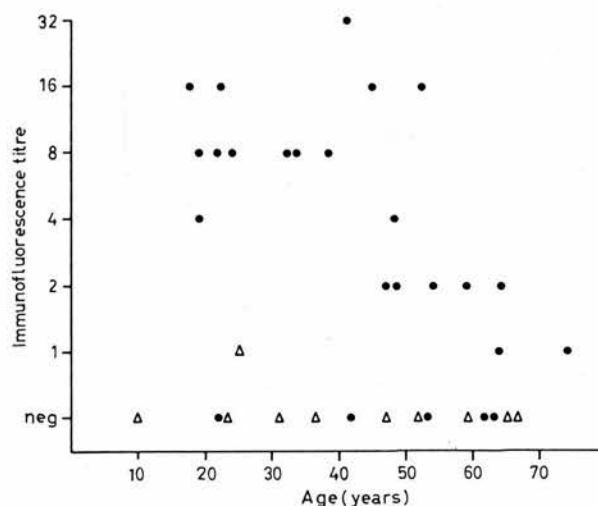


FIG. 7. The titre of adrenocortical antibody in the sera of patients with idiopathic and probable idiopathic adrenocortical insufficiency correlated with the patient's age at the time of serological study.  $\Delta$ , Male;  $\bullet$ , female.

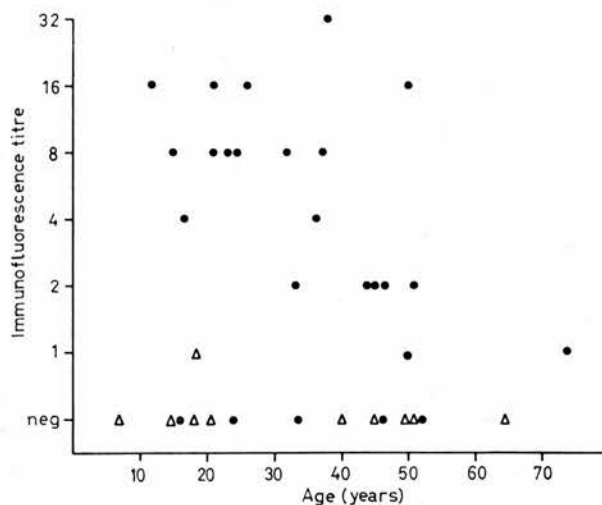


FIG. 8. The titre of adrenocortical antibody in the sera of patients with idiopathic and probable idiopathic adrenocortical insufficiency correlated with the patient's age at the time of diagnosis.  $\Delta$ , Male;  $\bullet$ , female.

Table 9 and Fig. 7 illustrate that the incidence and titre of adrenocortical antibodies is much higher in the female subjects with idiopathic or probable idiopathic adrenal insufficiency than in the male subjects in these disease groups. The incidence of adrenal antibody in

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patients with idiopathic or probable idiopathic adrenal insufficiency was 80% in the twenty-five female subjects compared to only 10% in the ten male subjects.

There is little or no correlation between the titre of adrenocortical antibody in the serum and the age of the patients with idiopathic or probable idiopathic adrenal insufficiency at the time of serological study (Fig. 7) or with the age of the patients at the time of diagnosis of adrenal insufficiency (Fig. 8). A few elderly patients appear to have lower titres than the

TABLE 9. Sex incidence of adrenocortical antibodies in adrenal insufficiency

Disease group	Sex	Adrenocortical antibodies	
		Positive	Negative
Idiopathic	F	9	0
	M	1	2
Probable idiopathic	F	11	5
	M	0	7
Both groups combined	F	20	5
	M	1	9

younger patients, but there is no correlation between the titres of adrenocortical antibody and the interval of time between the date of diagnosis of adrenal insufficiency and of obtaining a sample of blood for serological study (Fig. 9).

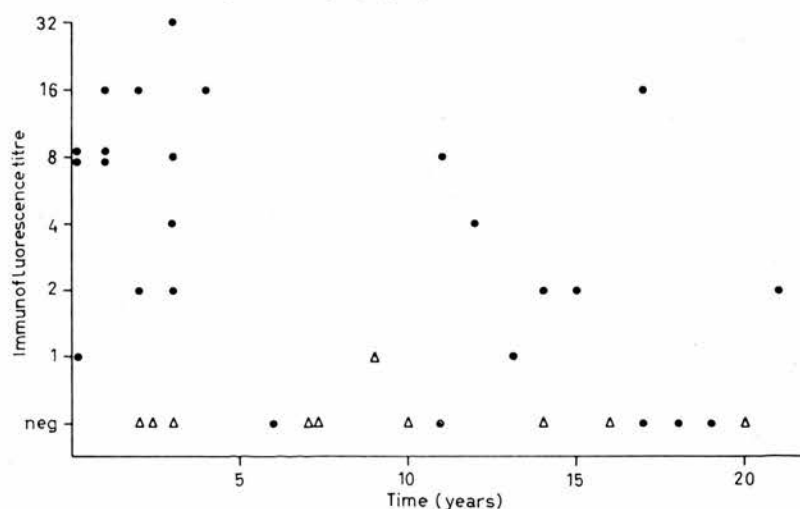


FIG. 9. The titre of adrenocortical antibody in the sera of patients with idiopathic and probable idiopathic adrenocortical insufficiency correlated with interval between time of clinical diagnosis and serological study. Δ, Male; ●, female.

The cord blood of the baby of patient No. 13 with probable idiopathic adrenal insufficiency contained an identical titre of adrenal antibody to that in the patient herself. The cord blood of patient No. 32, who also had probable idiopathic adrenal insufficiency was negative for adrenal antibody in keeping with the negative result in the patient's blood.

Adrenal antibody was found in the serum of four out of sixty-nine consecutive patients with lymphadenoid goitre attending the Endocrine Clinic. One of these patients (M.A. female 55 years) was also suffering from rheumatoid arthritis of longstanding for which she was being treated with phenylbutazone. Her blood pressure was 116/70 mmHg. There was no buccal pigmentation but there was vitiligo on the arms. A second patient (B.E., female, 65 years) had pernicious anaemia as well as histologically proven lymphadenoid goitre. A third patient (C.B., female, 58 years) had pernicious anaemia, diabetes mellitus, rheumatoid arthritis and clinical and serological evidence of lymphadenoid goitre. The fourth patient (M.B., female, 49 years) had histologically proven lymphadenoid goitre but was not known to be suffering clinically from any other abnormality at the time of writing, although parietal cell antibodies as well as thyroid and adrenal antibodies had

TABLE 10. Immunological survey in primary adrenal insufficiency

Type of adrenal disease	No. of patients	Sera positive for antibodies to:									A.N.F.	
		Thyroid			Stomach			Thyroid or stomach	Thyroid, stomach or adrenal	IgG		
		Tg.	Micr.	Total	P.C.	I.F.	Total					
Idiopathic	12	2	7	7	6	3	7	9	11	—	—	
Probable idiopathic	23	4	9	10	8	4	9	12	16	—	1	
Tuberculous	16	1	2	2	1	—	1	3	3	—	—	
Control subjects (matched for age and sex)	51	2	4	5	4	—	4	8	8	—	—	

Tg = Thyroglobulin; Micr = microsomal; P.C. = parietal cell; I.F. = intrinsic factor; adrenal = adrenocortical secretory cells.

been demonstrated in her serum. In the first three of these patients a  $\beta_{1-24}$  corticotrophin (Synacthen) test (Wood *et al.*, 1965) showed a normal level and reserve of adrenocortical function.

Adrenal antibody was also found in the serum of one of ninety-three patients with diabetes mellitus. The patient was a male aged 37 years and his serum was positive for gastric parietal cell antibody but negative for antibody to intrinsic factor and for thyroid cytoplasmic antibody. The adrenal function of these latter two patients has not yet been tested. The sera of eighty-nine patients with thyrotoxicosis, eighty-seven with primary atrophic hypothyroidism and fifty-four with pernicious anaemia were negative for adrenal antibody.

#### *Incidence of other antibodies in patients with adrenal sufficiency*

Table 10 summarizes the incidence of thyroid and gastric specific antibodies and of antinuclear factors in the population studied. Only one patient out of the fifty-one with adrenal insufficiency had antinuclear factor and this was in low titre and of IgM characteristic. The patient (No. 15) was a female aged 24 years who also suffered from hypoparathyroidism and moniliasis and who gave a family history of rheumatoid arthritis.



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There was a high incidence of thyroid and gastric antibodies in the patients with idiopathic or with probable idiopathic adrenal insufficiency. Antibody to thyroid cytoplasm was more frequently present than antibody to thyroglobulin. Antibody to intrinsic factor also had a high incidence in the idiopathic and in the probable idiopathic groups; seven out of thirty-five patients in these combined groups had antibody to intrinsic factor. Intrinsic factor antibody was not detected in any of the sera of the sixteen patients with tuberculous adrenal insufficiency or of the fifty-one control subjects. Eleven out of twelve patients with idiopathic and sixteen out of twenty-three patients with probable idiopathic adrenal insufficiency had one or more organ-specific antibodies to adrenal, thyroid or stomach. In contrast, the patients with tuberculous adrenal insufficiency had a much lower incidence (three out of sixteen) of organ-specific antibodies of the type studied and this incidence was comparable to that observed in the control subjects.

TABLE 11. Diseases associated with primary adrenal insufficiency

	Type of adrenal insufficiency		
	Idiopathic	Probable idiopathic	Tuberculous
Thyroid disease			
Thyrotoxicosis	1	2	—
Hypothyroidism	1	2	—
Euthyroid goitre	—	1	—
Pernicious anaemia	2	1	—
Hypoparathyroidism	1	2	—
Moniliasis	—	3	—
Diabetes mellitus	2	5	—
Rheumatoid arthritis	—	1	—
Asthma	1	2	—
Eczema	—	1	—
Schilder's disease	1	—	—
Alopecia totalis	—	1	1
Total affected	8	13	1
Total patients	12	23	16

*Clinical and subclinical disease of other tissues in patients with adrenal insufficiency*

The incidence of other clinical disorders in the patients with adrenal insufficiency is summarized in Table 11. The incidence of thyroid diseases is particularly remarkable. The diagnosis of disordered thyroid function was established in each case by clinical assessment and objective study using  $^{131}\text{I}$  uptake tests and serum PBI estimation, except in the one case (No. 28) indicated by a query in Table 3. The patient with the euthyroid goitre (No. 32) had a diffuse soft enlargement of the gland that was obvious on inspection and readily palpable and was estimated to be about 40 g in size. The serum protein bound iodine and radio-iodine uptake tests were normal.

One of the twelve patients with idiopathic adrenal insufficiency (No. 12) had evidence

of latent pernicious anaemia. The serum vitamin B<sub>12</sub> level was low at 108 pg/ml, the Schilling test of vitamin B<sub>12</sub> absorption gave an equivocal result at 8.9% (normal  $\geq 12.5\%$  using 0.5  $\mu\text{g}/0.5 \mu\text{c}$  <sup>58</sup>Co vitamin B<sub>12</sub>) and achlorhydria was demonstrated. A gastric biopsy contained no parietal cells and there was a degree of lymphocytic infiltration in the gastric mucosa that was considered greater than normal. Using the histological grading previously described (Irvine *et al.*, 1965) this patient's gastric biopsy was classified as P $\pm$  L+++. Another patient in this group (No. 8) had, in all probability, overt pernicious anaemia. The details about this patient are given under case No. 1 of the post-mortem findings. A third patient (No. 20) is of particular interest. Her serum had been sent from another hospital and was found to contain antibody to intrinsic factor in high titre as well as antibody to adrenal and antibodies to thyroid but not antibody to gastric parietal cells. While these studies were in progress, the patient was found to have a macrocytic blood picture. Subsequent investigation demonstrated a histamine fast achlorhydria (lowest pH, 7.7). The gastric secretion of intrinsic factor was 8.0 ng units in the basal hour and 6.0 ng units in the post-histamine hour establishing a diagnosis of Addisonian pernicious anaemia (Irvine, 1966a).

Three patients (Nos. 2, 15 and 20) with hypoparathyroidism were noted among the thirty-five with idiopathic or probable idiopathic adrenal insufficiency. Two of these patients also had moniliasis affecting the mouth, nails and vagina. The diagnosis of hypoparathyroidism was established by the clinical features, repeatedly low serum calcium levels, Ellsworth-Howard tests and response to therapy with calcium and vitamin D or A.T10 supplements. The other patient with moniliasis (No. 32) had suffered from recurrent attacks of oral and vaginal infection but had normal parathyroid function as determined by serial calcium estimations during a low calcium diet with the addition of sodium phytate (Smith, Davies & Fourman, 1960).

Seven out of thirty-five patients with idiopathic or probable idiopathic adrenal insufficiency also had diabetes mellitus. All seven patients were receiving treatment with insulin and the doses ranged from 20 to 72 units daily (average 42 units). One of the patients (No. 34) had diabetic retinopathy and neuropathy, but the incidence of diabetic complications in other patients was not established.

It was noted that one patient (No. 30) with probable idiopathic adrenal insufficiency had clinical, radiological and serological features consistent with mild rheumatoid arthritis. Two patients (Nos. 6 and 13) had adult onset asthma, one patient (No. 16) had asthma and eczema as a child, one (No. 11) had Schilder's disease and one (No. 20) had alopecia totalis. A full description of the patient with Schilder's disease is to be reported by Forsyth and Ingram (In preparation).

The only one of these conditions that occurred in the sixteen patients with tuberculous adrenal insufficiency was alopecia totalis (No. 46). There is, therefore, a striking difference in the incidence of associated diseases of the type listed in Table 11 among the patients with idiopathic or probable idiopathic adrenal insufficiency as compared to patients with tuberculous adrenal insufficiency.

A number of patients out of each of the three groups with adrenal insufficiency were studied for evidence of subclinical gastritis. The results are shown in Table 12. The incidence of achlorhydria and hypochlorhydria was significantly higher in the patients with idiopathic or probable idiopathic adrenal insufficiency than in the tuberculous group. The patient with latent pernicious anaemia (No. 12) and the two patients with frank pernicious

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anaemia (Nos. 8 and 20) are included among the eleven patients with idiopathic or probable idiopathic adrenal insufficiency who were shown to have achlorhydria. The other eight patients who were demonstrated to have achlorhydria had normal vitamin B<sub>12</sub> levels in the serum and in the four instances that a Schilling test was done the result was normal. The Schilling test was repeated 2 years later in one patient (No. 2) with antibody to intrinsic factor in the serum and the result was again within the normal range (39% and 23%, respectively). Quantitative figures for intrinsic factor secretion at these two intervals of time are not available. A gastric biopsy was obtained from eighteen patients out of the fifty-one with adrenal insufficiency; in general, the results correlated well with the figures obtained for the gastric acid secretion. The concentration of parietal cells varied inversely with the acid secretion in response to histamine and the degree of lymphocytic infiltration was noted to be increased in patients with achlorhydria and hypochlorhydria as compared to those with a normal acid secretion.

TABLE 12. Gastric function in primary adrenal insufficiency

Type of adrenal insufficiency	Gastric acid secretion in post histamine hour (mEq HCl)		
	Achlorhydria	> 0 < 5	≥ 5
Idiopathic	6*	2	1
Probable idiopathic	5	4	4
Tuberculous	—	2	5

\* Including three patients with pernicious anaemia.

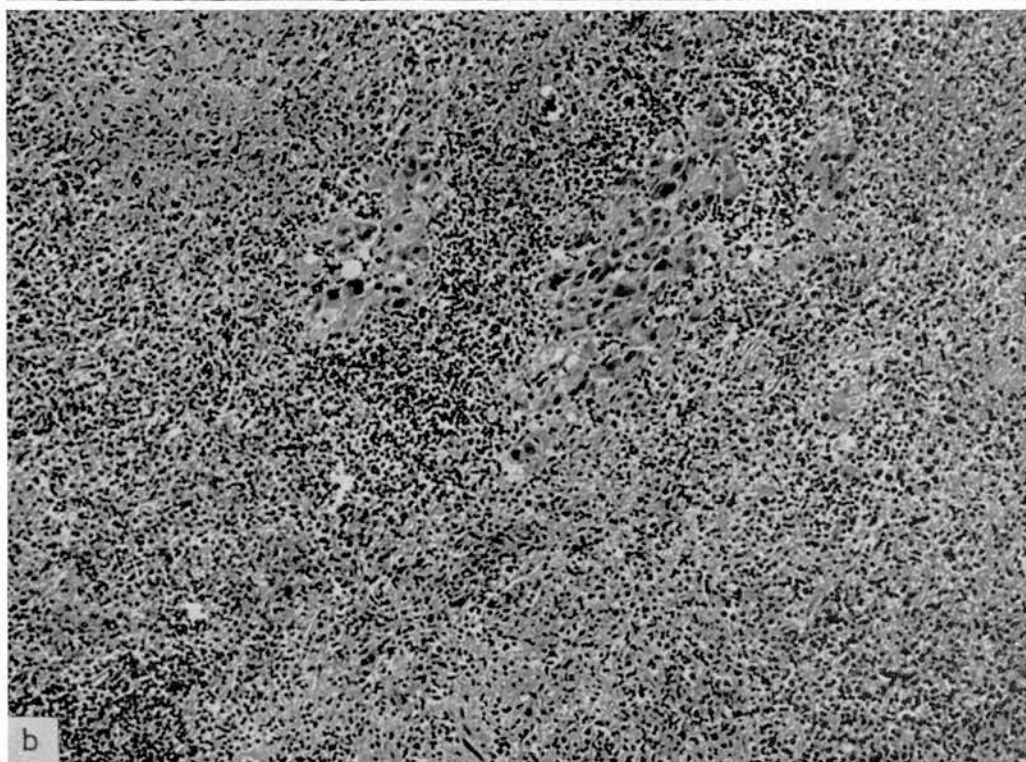
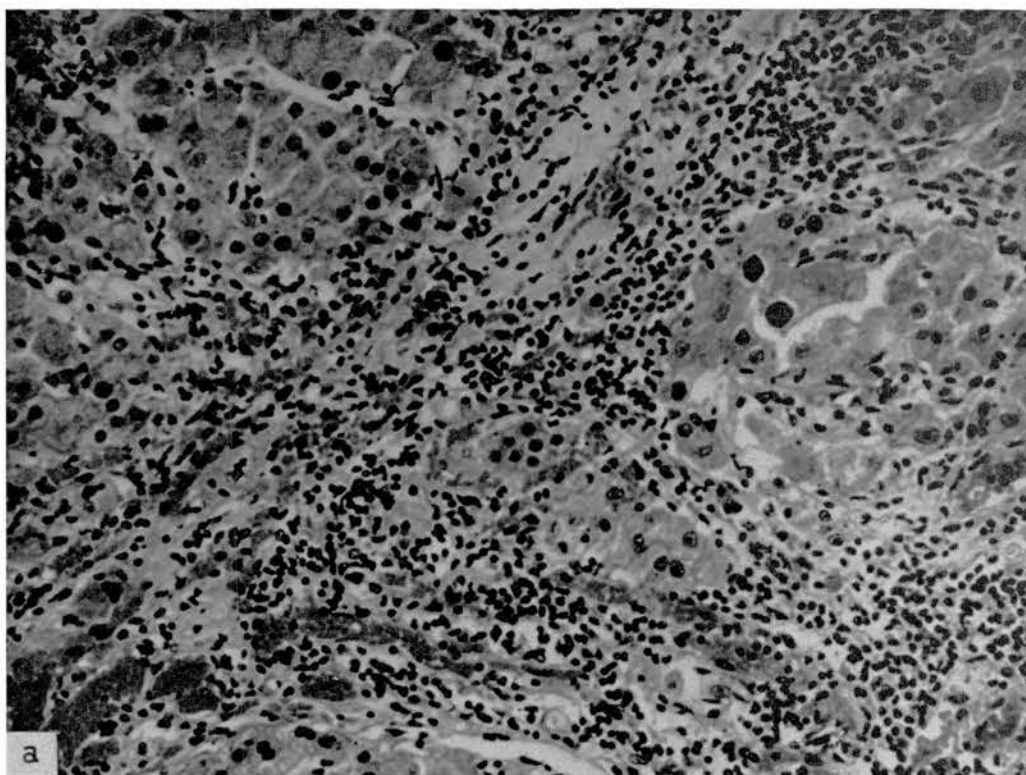
The level of protein bound iodine in the serum was measured in fourteen out of the thirty-five patients with idiopathic or probable idiopathic adrenal insufficiency who did not have any personal history of thyroid disease and the results were found to be normal. The level of protein bound iodine in the serum of twelve of the patients with tuberculous adrenal insufficiency who were so tested was also found to be normal. TSH stimulation tests to determine the reserve of thyroid function were not included in the present study.

*Family history of relevant diseases*

The history in the families of patients with adrenal insufficiency of diseases listed in Table 11 and of other diseases that may be associated with disordered immunological reactions is given in Table 13. The brother of patient No. 11 was reported to have died of Addison's disease. While there is a higher incidence of these diseases in the families of patients with idiopathic and probable idiopathic adrenal insufficiency than in the families of patients with tuberculous adrenal insufficiency, the difference is not striking and is of doubtful significance.

The family history of patient No. 24 is of particular interest. Several of her maternal relatives had diabetes mellitus and rheumatoid arthritis. Her mother was found to have primary hypothyroidism (typical clinical features, serum protein bound iodine = 1.0 µg/100 ml, serum cholesterol = 420 mg/100 ml) at this clinic 7 years ago. It may be pertinent that her father died from carcinoma of the stomach in view of the increased incidence







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of gastric carcinoma that is known to occur in pernicious anaemia (Kaplan & Rigler, 1945; Mosbeck & Videbaek, 1950).

Sera were studied from three close relatives of a patient with idiopathic adrenal insufficiency (No. 5) whose serum was positive for antibody to adrenal. None of these relatives had demonstrable adrenal antibody. Six relatives of three patients with idiopathic or probable idiopathic adrenal insufficiency (Nos. 11, 24 and 32) who did not have detectable adrenal antibodies were likewise negative in this respect. Four of the total of nine relatives had antibody to gastric parietal cells but all were negative for antibody to intrinsic factor. One serum was positive to thyroglobulin.

TABLE 13. Family history of diseases in patients with adrenal insufficiency

	Type of adrenal insufficiency		
	Idiopathic	Probable idiopathic	Tuberculous
Addison's disease	1	—	—
Thyroid disease	2	5	2
Pernicious anaemia	1	2	—
Hypoparathyroidism	—	—	—
Moniliasis	—	1	—
Diabetes mellitus	2	6	3
Rheumatoid arthritis	2	3	—
Asthma	2	4	2
Coeliac disease	—	1	—
Bright's disease	—	—	1
Total affected	5	12	5
Total patients	12	23	16

*Autopsy reports*

Two cases of adrenal insufficiency coming to autopsy were available for study.

(1) D.M., case No. 7 of Tables 1 and 5. A Caucasian female aged 74 years. This patient was admitted to hospital with a 3-month history of vomiting, weakness and central abdominal pain. During the previous year she had lost 14 kg in weight. Some 30 years previously she was thought to have had a syphilitic labyrinthitis. Eleven years previously a macrocytic anaemia and histamine fast achlorhydria were demonstrated. There were no symptoms of intestinal malabsorption and the patient was not known to be taking any

FIG. 10. (a) The histology of the adrenal gland of the patient D.M. aged 74 years who died with idiopathic adrenal insufficiency (case No. 8 of Tables 2 and 5; autopsy report No. 1). Note the disruption of the normal architecture of the adrenal cortex and the infiltration of the tissue with lymphocytes. H & E,  $\times 360$ .

(b) The histology of the adrenal gland of the patient R.M. male aged 63 years who died with idiopathic adrenal insufficiency (autopsy report No. 2). Note the diffuse and heavy infiltration of the gland epithelial cells. H & E,  $\times 125$ .

drugs that might have induced a macrocytic blood picture. Although no bone marrow, serum vitamin B<sub>12</sub> or absorption studies were done, a diagnosis of pernicious anaemia was made and the patient responded well to treatment with parenteral vitamin B<sub>12</sub>. The patient continued to receive regular injections of vitamin B<sub>12</sub> during the remainder of her life.

The skin was markedly pigmented, particularly the backs of the hands, creases and pressure areas. Buccal pigmentation was also present. The blood pressure was 110/75 mmHg. The haemoglobin was 70%, the blood film and indices were normocytic and the reticulocyte count was 1%. The serum sodium concentration was below normal limits at 127 mEq and the serum potassium and bicarbonate concentrations were within normal limits at 4.5 and 25 mEq, respectively. No information was available with regard to urinary or plasma steroid levels.

Shortly after admission the patient collapsed with a blood pressure of 80/40 mmHg and in spite of treatment with hydrocortisone and desoxycortisone acetate the hypotension persisted and the patient died following a series of fits 5 days later.

At autopsy (Dr Beswick) both adrenal glands were very small, the right being especially minute (left adrenal = 3.5 g, right adrenal = 2.0 g together with some adherent fat and connective tissue). The adrenal cortices were very narrow and depleted of lipid. Microscopy of the left adrenal showed that the normal cortical layers could not be made out. The surviving cortex consisted of nodular masses of large irregularly-arranged palely-eosinophilic cells. In places cortical tissue was completely absent and replaced by vascular fibrous tissue. Lymphocytic clusters were very numerous through the gland (Fig. 10a). The right adrenal showed almost no glandular tissue whatever except for one small area of cortical cells infiltrated with lymphocytes. Elsewhere the layers of the thickened capsule were separated only by a little delicate connective tissue infiltrated by clusters of lymphocytes. The arteries in both adrenal glands were somewhat thick-walled and had prominent elastic laminae. Some of the arterioles were hyalinized and the veins had thickened muscular walls.

The pituitary was of normal size. On histological examination the anterior lobe of the pituitary contained a few small fibrous scars and a cluster of lymphocytes. The thyroid appeared grossly normal and weighed 20 g. Histologically the thyroid was somewhat nodular and the vesicles varied in size but contained a normal content of colloid. In some areas there was some patchy fibrosis and lymphocytic infiltration in the thyroid but this was not conspicuous. The parathyroid glands were not examined. The pancreas showed no abnormality. No thymus remnants were seen.

There was extensive bronchopneumonic consolidation in all lobes of both lungs and histological examination showed congestion and an acute bronchitis. There was no evidence of tuberculosis anywhere in the body nor was there any evidence of syphilitic infection. In particular, the middle ears were normal. There was no gross abnormality seen in the central nervous system apart from congestion and oedema and examination of the cardiovascular system was not remarkable. The histology of the stomach was vitiated by the presence of autolysis.

A sample of serum was obtained a few days before her death. It contained a low titre of adrenocortical antibody both by the indirect fluorescent antibody technique and by complement fixation. The serum also contained antibody to thyroid microsomal antigen (positive immunofluorescence; complement fixation titre 1:32) and antibody to intrinsic factor in a titre of 39 ng/ml. The serum was negative for antibody to thyroglobulin and for

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antibody to gastric parietal cell cytoplasm and for anti-nuclear factors and did not give rise to non-specific autoimmune complement fixation reactions. Unfortunately, no unfixed tissue was available for direct immunofluorescence studies.

(2) R.M., 63-year-old Caucasian male with a 3-month history of lassitude and weight loss. Clinical examination revealed only evidence of weight loss. Blood pressure = 105/65 mmHg, haemoglobin = 70%. On two occasions the erythrocyte sedimentation rate was noted to be raised at 30 and 50 mm in the first hour (Westergen). Malignancy was suspected. X-ray of chest, barium meal and barium enema showed no abnormality. No definite diagnosis was made and the patient died at home. No serum was available for immunological study.

At autopsy (Dr Neil Maclean) the only macroscopic abnormality was in the adrenal glands; they were below average size (left adrenal = 6.0; right adrenal = 5.4) and sectioning revealed that very little cortical tissue remained. Much of the gland was composed of firm grey tissue. There was no macroscopic evidence of tuberculosis elsewhere in the body. The pituitary and thyroid were of normal size (pituitary weight = 0.6 g). The testes, pancreas and liver (1360 g) were considered to be below average size but no abnormality was noted on sectioning of these organs, apart from a mild degree of brown atrophy in the liver. The spleen weighed 140 g and showed no abnormality. No reference was made to the thymus or parathyroids. The sternum, vertebrae and right iliac crest showed some osteoporosis and these bones contained dark red reactive marrow.

On microscopic examination the adrenal glands were seen to be almost wholly destroyed. Here and there surviving cortical and medullary cells were still recognizable but they formed only a small proportion of the total gland. There was a heavy degree of lymphocytic infiltration, but plasma cells were also present (Fig. 10b). In addition there were areas of necrosis and fibrosis. No multi-nucleated giant cells or tubercle bacilli were identified but epithelioid cells surrounded some of the necrotic areas. No excess of iron pigment could be demonstrated in the adrenals.

There was no microscopic evidence of tuberculosis elsewhere in the body. Minor siderotic lesions of the lungs had developed as a result of the patient's occupation of arc-welder. The gastric mucosa showed some degree of lymphocytic infiltration together with large lymphoid follicles but parietal cells were plentiful. Detailed analysis of the gastric histology was prevented by autolysis. The thyroid, pancreas and pituitary were histologically normal. The only other abnormality noted was the presence of foci of myofibrillar atrophy and of associated lymphocytic infiltration in a portion of pectoral muscle.

## DISCUSSION

A diagnosis of adrenal insufficiency necessitates the appraisal of the clinical features which the patient presents together with the appropriate investigation of the patient's adrenal function. The most specific test for adrenal insufficiency is the level of the plasma cortisol and the absence of a response in this level to adrenal stimulation by ACTH or  $\beta_{1-24}$  corticotrophin (Synacthen, Ciba). The ideal time to do this investigation is when the diagnosis of adrenal insufficiency is first suspected. Frequently, however, the diagnosis is made clinically and in fear of impending adrenal crisis steroid therapy is commenced. The patient improves clinically and further investigation may not be instituted. Particular care must be taken to use ACTH in adequate dosage and for sufficient time in the assessment of



potential adrenal function in a patient who has been treated with steroids for a prolonged period. In such a patient a rise in the plasma 11-OHCS level may be obtained with Synacthen stimulation, but lack of response should be followed by adequate ACTH testing. We have shared the common experience of studying patients who were initially diagnosed on the strength of the clinical features of Addison's disease, but who on investigation with plasma cortisol studies before and after ACTH were subsequently shown to have normal adrenal function. For the purpose of scientific study clinical features alone are not in themselves sufficient to label the patient as having adrenal insufficiency; patients diagnosed on clinical features alone or together with laboratory tests that are not specific for primary adrenal failure, such as urinary ketosteroid excretion, water tolerance, glucose tolerance, insulin sensitivity, etc., should be placed in a category of 'probable adrenal insufficiency'.

The diagnosis of idiopathic adrenal insufficiency is made by the process of exclusion. Among the known agents that may cause adrenal destruction are tuberculosis and rarely histoplasmosis, sarcoidosis, syphilis, amyloidosis, haemorrhage, infarction, mycosis fungoides, Hodgkin's disease or tumour. If the patient has radiological calcification of the adrenal glands it may be assumed that the aetiology is other than idiopathic. We are not aware of any description in the literature of calcification of the adrenal that has been the site of simple atrophy. While tuberculosis is the commonest cause of adrenal calcification it is conceivable that this might also be the result of some other pathology, e.g. haemorrhage or infarction. The proportion of tuberculous adrenal glands that show radiological evidence of calcification is not known. Figures from the older literature (Camp, Ball & Greene, 1932) are probably not applicable to the present day. Prior to satisfactory treatment of adrenal insufficiency with steroid replacement, active tuberculosis may have destroyed sufficient cortical tissue to cause death before fibro-caseation or calcification occurred. With survival of the patient the incidence of calcification of the adrenal is likely to be higher, particularly in patients who have been X-rayed many years after adrenal insufficiency was diagnosed. In the absence of adrenal calcification it would seem logical to assume a tuberculous aetiology if the patient has evidence of gross tuberculous infection elsewhere in the body either preceding or coincident with the diagnosis of adrenal insufficiency. The development of gross tuberculous infection after the diagnosis of adrenal insufficiency could be due to the possibility that patients with adrenal insufficiency of whatever origin may be more prone to tuberculous infection. Nine out of the sixteen patients in the tuberculous group in the present study had radiological evidence of adrenal calcification.

A positive Wassermann reaction does not necessarily mean that the patient's adrenal insufficiency is syphilitic in origin and a positive Mantoux test or radiological opacity on chest X-ray does not necessarily mean that this adrenal insufficiency is due to tuberculosis. This is exemplified by patient No. 8 who was known to have a positive Wasserman reaction but who had no evidence of syphilis at autopsy and whose adrenal glands showed simple atrophy. At the present time in Britain radiological evidence of the healed primary tuberculous focus in the chest is a common occurrence in middle-aged and elderly persons. It would therefore not be judicious to label adrenal insufficiency as tuberculous in origin simply because there was a healed primary focus on chest X-ray or a positive Mantoux reaction. For this reason we would not agree with the subdivision of patients with adrenal insufficiency into idiopathic or tuberculous according to the presence or absence of radiological evidence of tuberculosis anywhere in the body as used by Goudie *et al.* (1966).



The incidence of adrenocortical antibodies in the different subgroups of patients with primary adrenal insufficiency will depend not only on the diagnostic criteria used to classify the patients but also on the techniques employed for the detection of these antibodies. Antibody to adrenal can be detected using the methods of immunofluorescence and complement fixation. The sensitivity of the immunofluorescence method depends on the details of the procedure adopted. With weak anti-sera, inappropriate conjugation with the fluorescent dye, inadequate tissue sections or insufficient washing after application of the conjugate, the background non-specific staining of the adrenal tissue may be so dense as to mask even strong specific staining. In the present study the most satisfactory results were observed with anti  $\beta_{1C}$  conjugated to fluorescein iso-thiocyanate. It should also be noted that, although the zona glomerulosa is readily recognizable and prominent in most human adrenal glands, it may not be present in all sections and may exist only as small focal collections of cells on the surface of the gland. In the sera of patients with idiopathic or probable idiopathic adrenal insufficiency a good correlation was observed between the titre of adrenocortical antibody as determined by immunofluorescence and by the complement fixation method. One serum from a patient with tuberculous adrenal insufficiency gave a positive complement fixation test with adrenal and failed to stain the secretory cells of the adrenal cortex in the immunofluorescence method. Blizzard & Kyle (1963) record a similar finding with the sera of two patients who were believed to have Addison's disease of tuberculous rather than idiopathic origin. The fact that this serum gave strong staining of the vascular endothelium with anti- $\beta_{1C}$ -FITC conjugate suggests that the positive complement fixation reaction with a saline extract of adrenal was directed against the interstitial tissue of adrenal and not the secretory cells. The staining of the interstitial tissue (vascular endothelium) of adrenal gland No. 1 is probably related to blood group A substance, although this has not been definitely established. Its occurrence is of importance, at least in a negative sense, in that such reactions may be responsible for 'false positive' complement fixation tests with saline extracts of adrenal.

The adrenocortical antibody is IgG in character as determined by the use of monovalent antisera in the immunofluorescent technique and by the observation that the antibody was able to cross the placenta into the foetal circulation.

Blizzard & Kyle (1963) give some evidence that suggests there may be more than one antibody to adrenal as detected by complement fixation. The observation that the pattern of immunofluorescent staining of adrenal sections with the sera of patients with idiopathic adrenal insufficiency is the same when anti-IgG or anti- $\beta_{1C}$  conjugates were used suggests that, if there are two antibodies to adrenal, the corresponding antigens have a similar distribution within the gland. It is difficult but possible to grow human adrenal cells in tissue culture (Irvine, 1960) and it would be interesting to correlate cytotoxicity experiments with the findings obtained with immunofluorescence and complement fixation in a manner comparable to what has been done in relation to the problem of thyroiditis (Forbes *et al.*, 1962; Irvine, 1962; Kite *et al.*, 1965). The adrenocortical antigen is a particulate component of the cytoplasm and is found predominantly in the microsomal but also in the mitochondrial fractions of a saline extract (Blizzard & Kyle, 1963; Goudie *et al.*, 1966). It is interesting to speculate why the staining should be of maximal intensity in the zona glomerulosa. A number of steroid hormones are formed by the adrenal cortex, the most important of which are the  $C_{21}$  steroids, cortisol, corticosterone and aldosterone. Cortisol and

corticosterone are products of the reticularis and fasciculata zones, while aldosterone is formed by the zona glomerulosa. The adrenal cortical cells also form  $C_{19}$  compounds or androgens and opinions vary as to the site of production of these hormones. Greep & Deane (1949) demonstrated that injections of corticosterone into the intact rat result in depletion of the sudanophilic material in the zona fasciculata and reticularis in a manner comparable to the changes seen following hypophysectomy, while the lipids in the zona glomerulosa remained essentially unaffected. It is conceivable that the content of adrenal antigen in the zona fasciculata and reticularis was depleted in the glands which we studied as a result of the administration of exogenous corticosteroid to the patient prior to removal of the adrenals. On the other hand, the occurrence of maximum intensity of fluorescent staining in the zona glomerulosa might be related to the type of metabolism that is characteristic of this layer of adrenal cortex. It is possible that immunological techniques may prove helpful in analysing the different constitution of the zona glomerulosa compared to the zona fasciculata and reticularis. The negative results of adrenal positive sera when tested against sections of human testis indicate that the antigen in the adrenocortical cells is not common to all steroid producing tissues.

No one has yet demonstrated that adrenocortical antibody in patients with adrenal insufficiency is an autoantibody and strictly speaking it would seem unwise to refer to these antibodies as such until this point has been established. However, the similarity in the immunological phenomena associated with thyroiditis (Roitt & Doniach, 1960), atrophic gastritis (Irvine *et al.*, 1965) and idiopathic adrenal insufficiency would make it probable that the adrenocortical antibody is an autoantibody.

The incidence recorded in this study of adrenocortical antibodies in the sera of patients with idiopathic adrenal insufficiency is very high, although it is admitted that the number of patients in this group is small. All the female patients in this group were positive and one of the three male patients in the group was positive. Even when the patients with probable idiopathic adrenal insufficiency are included, the incidence of adrenocortical antibody is still high in the female subjects at 80%. The titre of adrenal antibody in the serum of the one positive male patient of the ten with idiopathic or probable idiopathic adrenal insufficiency was minimal. The sex distribution of the patients with idiopathic or probable idiopathic adrenal insufficiency was predominantly female, while in the patients with tuberculous adrenal insufficiency it was predominantly male. It is frequently stated that the sex incidence in adrenal insufficiency is approximately equal (Sorkin, 1949; Thorn, Dorrance & Day, 1942; Dunlop, 1963; Goudie *et al.*, 1966), but on analysis of these reports there has been no or inadequate distinction made between adrenal insufficiency of tuberculous or idiopathic origin. In Thorn's series, while there was an equal sex incidence in patients with idiopathic adrenal insufficiency assessed clinically, of the fourteen patients with adrenal insufficiency who came to autopsy, seven had simple atrophy of the adrenal cortex and of these six were female. In the autopsy cases of simple adrenal atrophy reported by Brenner (1928) and by Susman (1930) there were eight females and two males. The preponderance of females among adult patients with idiopathic adrenal insufficiency is in keeping with the criteria of an 'autoimmune disease'. The sex ratio may not be so pronounced, however, in favour of females as it is in thyroiditis or Addisonian pernicious anaemia. The preponderance of adrenal antibody positive subjects among females, with a low incidence among males, is not in keeping with thyroiditis or Addisonian pernicious

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anaemia for, although these diseases occur predominantly in females, males who do develop these conditions tend to have the same incidence of thyroid and gastric antibodies as the females. As in thyroiditis and gastritis so also in adrenalitis, there is no correlation between the titre of the corresponding organ-specific antibody and the duration of the clinical disease in a population of patients studied at one point in time.

The titres of adrenal antibody so far encountered ( $\leq 1:32$ ) are not high. The range is less than that for parietal cell antibody in the sera of patients with pernicious anaemia and certainly less than that for thyroid complement fixing antibody in patients with chronic thyroiditis. In keeping with this fact is the absence of any gross quantitative abnormality in the  $\gamma$ -globulin fraction of the serum as determined by cellulose acetate electrophoresis and the normal to slightly elevated levels of the erythrocyte sedimentation rate in patients with idiopathic adrenal insufficiency.

The high incidence of other endocrinopathies (thyroid disease, hypoparathyroidism, diabetes mellitus) and of atrophic gastritis in patients with idiopathic but not tuberculous adrenal insufficiency is of interest. Schmidt (1926) was the first to describe two cases with the symptoms of idiopathic adrenocortical insufficiency accompanied by lymphocellular infiltration and germinal centres in the thyroid. Carpenter *et al.* (1964) reviewed the literature on the association between thyroid and adrenal insufficiency and reported a further fifteen cases, including ten instances of coexistent diabetes mellitus. As in our own experience, thyroid changes were found much more commonly in patients with idiopathic adrenal insufficiency than in patients with adrenal tuberculosis. This observation was also made by Sloper (1953) who concluded that lymphoid infiltration in the thyroid of patients with simple atrophy of the adrenal cortex was not dependent on the general hyperplasia of the lymphatic tissue commonly associated with Addison's disease. The female to male ratio in Schmidt syndrome is approximately 3:1. The occurrence of thyrotoxicosis in patients with idiopathic adrenal insufficiency has previously been noted by Blizzard & Kyle (1963) and by Irvine (1963b). Sorkin (1949) noted at autopsy that idiopathic atrophy of the adrenal glands was present in twelve out of fourteen cases of combined Addison's disease and diabetes mellitus and that adrenal tuberculosis occurred in only two. Beaven *et al.* (1959) reviewed fifty-five cases in the literature of diabetes mellitus and Addison's disease and added a further eight; the majority of these patients had idiopathic rather than tuberculous adrenal insufficiency. Diffuse cerebral sclerosis with endocrine abnormalities has been described in young males by Hoefnagel, van den Hoort & Ingbar (1962). In their patient, there was a virtual absence of basophilic cells in the pituitary and the marked atrophy of the adrenal cortices which was also present may have been secondary to this. Blizzard & Kyle (1963) refer to a case of Schilder's disease associated with idiopathic adrenal insufficiency. The patient included in the present paper is another example. It is of interest that autoimmune phenomena may be present in demyelinating conditions. (Lamoureux & Borduas, 1966).

The association between juvenile hypoadrenocorticism, hypoparathyroidism and superficial moniliasis was first noted by Whitaker *et al.* (1956), and this association, particularly in the younger patients with idiopathic adrenal insufficiency, has now been frequently recorded (Blizzard & Kyle, 1963; Hung, Migeon & Parrott, 1963; Kunin *et al.*, 1963; Kenny & Holliday, 1964). The description of a possible association between pernicious anaemia and adrenal insufficiency dates back to Addison himself (Addison, 1849). The



association between pernicious anaemia and adrenal insufficiency had been reviewed by Kra & Barile (1964) and has also been noted in cases of idiopathic adrenal insufficiency by Hung *et al.* (1963) and by Morse, Cochrane & Landrisan (1961).

With regard to subclinical disease, atrophic gastritis associated with hypochlorhydria or achlorhydria has been described in Addison's disease (Smith, Delamore & Wynn Williams, 1961). The unsubstantiated statement has been made (Soffer, 1946; Sorkin, 1949) that the achlorhydria of adrenal insufficiency is reversible. The present study emphasizes that atrophic gastritis is particularly common and severe in patients with idiopathic adrenal insufficiency and is less common and less severe in patients with tuberculous adrenal insufficiency. It also indicates that at least in some cases the gastritis is not reversible for it has been shown that severe gastric atrophy with achlorhydria is commonly present many years after adequate steroid replacement therapy has been instituted and maintained in patients with idiopathic adrenal insufficiency.

A degree of lymphocytic infiltration of the pituitary was observed in autopsy No. 1 of the present study and this has been previously observed by Susman (1930) and by Carpenter *et al.* (1964). In autopsy No. 2, lymphocytic infiltration in skeletal muscle was noted. This, and also lymphocytic infiltration of the meninges, has been described by Duff & Bernstein (1933). In neither of the autopsies included in the present study was there any evidence of lymphocytic infiltration of the liver or pancreas, which has been noted to occur by Susman (1930).

In a review of the microscopic sections of the adrenal glands of twenty-eight patients found to have typical Hashimoto's disease at autopsy, Carpenter *et al.* (1964) found no evidence of lesions resembling the changes of idiopathic adrenal atrophy. In the records of autopsies in seventy-four patients with Hashimoto thyroiditis, Masi *et al.* (1965) could not find any evidence that atrophy or lymphocytic infiltration of the adrenal glands was any more common than that found in seventy-four control autopsies.

The incidence of other organic-specific antibodies is strikingly high in patients with idiopathic as opposed to tuberculous adrenal insufficiency. This is in keeping with the high incidence of associated clinical and subclinical conditions referred to above. The high incidence of antibody to intrinsic factor is particularly remarkable. In general, the presence of intrinsic factor antibody in the serum shows a very strong correlation with malabsorption of vitamin B<sub>12</sub> due to failure of secretion of intrinsic factor by the gastric mucosa or an inhibition of what little intrinsic factor is being secreted (Irvine, 1965; Fisher, Rees & Taylor, 1966). Seven out of the thirty-five patients with idiopathic or probable idiopathic adrenal insufficiency in the present study had antibody to intrinsic factor in the serum and yet only three had evidence of latent or overt pernicious anaemia. Tests for antibody to parathyroid tissue were not included in the present study. Blizzard, Chee & Davis (1966) described parathyroid antibodies in 38% of seventy-four patients with idiopathic hypoparathyroidism, in 6% of 245 control patients and in as many as 26% of ninety-two patients with idiopathic adrenal insufficiency. In keeping with the low incidence of Addison's disease in patients with lymphadenoid goitre, sera of four out of sixty-nine patients with lymphadenoid goitre were found to be positive for adrenocortical antibody, and all four of these patients were shown to have normal adrenocortical function. Likewise, the incidence of adrenocortical antibodies in consecutive patients with thyrotoxicosis, primary atrophic hypothyroidism, pernicious anaemia and diabetes mellitus and in control subjects was low (only one positive recorded out of 372 sera). It is possible that a certain type of diabetes mellitus may be associated with



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other endocrinopathies. Our findings do not offer many clues in this respect except that all our patients were receiving moderate to high doses of insulin and at least one had diabetic complications. Autoimmune phenomena to pancreas or insulin in diabetic subjects do not appear to be clearly established (Chetty & Watson, 1965). Mancini *et al.* (1965) have reported finding antibodies to insulin in two patients who had diabetes mellitus but who had not been treated with insulin. However, the number of controls in the latter study was very small.

On present evidence it may be stated that idiopathic adrenal insufficiency is part of the syndrome of polyendocrinopathy including the thyroid gland, parathyroid gland and the adrenal, and to which may be added atrophic gastritis and a tendency towards diabetes mellitus. From a review of the literature the disease complex may also include steatorrhea (Kunin *et al.*, 1963). This syndrome may be manifest clinically in one or more of its facets and there may be subclinical evidence of other facets of the syndrome. There is no fixed order in which the different tissues may be involved; one or more of these conditions may be clinically manifest before the adrenal insufficiency becomes apparent or the adrenal insufficiency may precede the others.

The rarity of idiopathic adrenalitis and of idiopathic hypoparathyroidism contrasts with the comparatively common occurrence of thyroid disorders and of atrophic gastritis. This fact requires some explanation if we are to argue that all these conditions have a common pathological mechanism. It does appear that, if a patient has thyroiditis, he has a good chance of having gastritis also and vice versa, but he has little chance of having adrenalitis or parathyroid insufficiency. On the other hand, if the patient has adrenalitis of idiopathic origin, he has a very good chance of having clinical or subclinical thyroid disease, or gastritis with the corresponding organ-specific antibodies in the serum. There is, therefore, some differential susceptibility of the target organs in this group of diseases. One obvious correlation is the size of the glands involved, but this may be too simple a basis on which to seek an explanation.

Familial aspects of Addison's disease have recently been reported by Hung *et al.* (1963). Meakin, Nelson & Thorn (1959) reviewed the literature and found five cases of familial Addison's disease documented by steroid studies or autopsy findings and reported another case. Dunlop (1963) drew attention to the fact that all cases of familial Addison's disease have so far proved to be idiopathic if autopsy was performed. While it is apparent from Table 13 that a family history of other endocrinopathies and of diseases associated, or possibly associated with, immunological abnormalities is a common feature in patients with idiopathic adrenal insufficiency, these diseases also occurred in the family histories of patients with tuberculosis of the adrenals. Thyroid disease, diabetes and asthma are all common conditions; a much larger series of patients would need to be studied before the suggestive findings reported here could be said to be adequately documented. A systematic approach to the problem is required whereby as many of the relatives as possible are interviewed and clinically examined.

The post-mortem studies on the two patients with idiopathic adrenal insufficiency described in the present paper emphasize the similarity of the histology of simple adrenal atrophy to that of other tissues (e.g. thyroid, stomach) which have been the site of a disease process that is associated with autoimmune phenomena. While germinal centres were not a feature, there was in both cases a heavy lymphocytic infiltration and a disruption of the

normal architecture of the gland and some increase in fibrosis. As noted in thyroiditis (Irvine & Muir, 1963) and gastritis (Irvine *et al.*, 1965), the lymphocytes can be seen under light microscopy to come into close relation with the adrenal epithelial cells. While the majority of patients with simple atrophy of adrenal have very small glands which are virtually fibrous and contain very few epithelial adrenal cells and some lymphocytic infiltration (autopsy No. 1), it is of interest to note that the adrenal gland may be of considerable size and very densely infiltrated with lymphocytes in a manner comparable to the enlarged but underactive Hashimoto goitre (autopsy No. 2). We believe that this is the first description of such a case. Unfortunately a sample of this patient's serum was not available for immunological study.

The immunology of adrenal insufficiency is of particular importance from the theoretical point of view. Here we have a distinct difference between adrenal insufficiency due to simple atrophy and that due to bacterial destruction. In the former there is a high incidence of organ-specific antibodies (including those directed against the adrenal) while in the latter these antibodies are virtually absent. This observation is of particular relevance when it is recalled that tubercle bacilli have an adjuvant effect on the immunological system. As previously argued (Irvine, 1964) there must therefore be some basic immunological difference between these two categories of patients with adrenal insufficiency. A disorder of immunological tolerance may be sited in the antibody forming system itself or possibly in the thymus. There is little in the literature on the pathology of the thymus gland in patients with adrenal insufficiency. An enlarged thymus gland was noted by Carpenter *et al.* (1964) in their survey of the autopsy findings of twenty-four patients with Addison's disease, but that particular patient had tuberculous disease of the adrenal. Duff & Bernstein (1933) noted the presence of a cystic mediastinal tumour in the region of the thymus during the autopsy on a patient with simple atrophy of the adrenals. Structures resembling germinal centres were noted to be present in the tumour but no Hassell's corpuscles were seen and the cystic spaces were filled with amorphous caseous material. In two other autopsies in their series the thymus gland was of normal appearance. Wells (1930) noted a 'persistent' thymus in only one of his five post-mortem cases of combined thyroiditis and suprarenal atrophy. Several factors might influence the size and histology of the thymus in adrenal insufficiency; a disorder of immunological tolerance in a manner comparable to thyroiditis (Gunn, Michie & Irvine, 1964; Irvine & Sumerling, 1965), the change in the steroid status of the patient and the existence of infection, particularly tuberculous infection.

Adrenalitis has been produced experimentally by immunizing guinea-pigs with homologous or autologous adrenal tissue and Freund's adjuvant (Colover & Glynn, 1958; Steiner *et al.*, 1960; Witebsky & Milgrom, 1962). There appeared to be no correlation between the titre of the adrenal antibodies as detected by complement fixation and the degree of histological aberration (Terplan, Witebsky & Milgrom, 1963). Some but not all the guinea-pigs injected with adrenal extract and Freund's adjuvant developed histological lesions in the adrenal gland. The interpretation of this observation was complicated by an occasional positive histological finding in the adrenals of control animals injected with adjuvant alone (Witebsky & Milgrom, 1962). These results are closely comparable to what has been observed in relation to experimental thyroiditis (Jones & Roitt, 1961). Comparable lesions of the gastric mucosa, parathyroids and pancreas have not been convincingly produced in experimental animals by immunological means. The adrenal antigen was shown to be

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probably protein in nature and associated with the 6.6 S sedimenting component (Shulman *et al.*, 1965).

Whatever the theoretical implications of the description of antibodies to the adrenal cortex in idiopathic adrenal insufficiency, the detection of these antibodies may have some clinical importance. The finding of adrenal antibody would suggest an idiopathic aetiology for Addison's disease that is already diagnosed or clinically suspected. There may be a place for screening patients with other endocrinopathies serologically to determine whether they may have a tendency to develop overt adrenal insufficiency at a later date although the number of positive results is likely to be small. As in the case of thyroiditis and gastritis, the detection of adrenal antibody may prove to be a pointer to subclinical disease of the adrenal gland. The finding of adrenocortical antibody in the serum of one out of ten patients with Cushing's syndrome together with a description of lymphocytic infiltration in hyperlastic human adrenal glands (Wegienka *et al.*, 1966) suggests that adrenal specific antibody may be associated with hyper- as well as hypo-function of the adrenal cortex in a manner comparable to thyroid autoimmunity in thyrotoxicosis, Hashimoto goitre and primary thyroid atrophy (Roitt & Doniach, 1960; Adams, 1965). Clearly a detailed clinical, immunological and histological analysis of a larger number of patients with different types of adrenal disorder is required.

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ADDISON'S DISEASE AND ADDISONIAN ANAEMIA

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Lancet (1967), i, 723-724.

## ADDISON'S DISEASE AND ADDISONIAN ANÆMIA

SIR,—In their article Dr. Meecham and Dr. Wyn Jones (March 11, p. 535) write: "It would be of interest to screen patients with adrenal insufficiency for evidence of vitamin-B<sub>12</sub> deficiency"

We have made a clinical and immunological study of 51 patients with Addison's disease (12 with idiopathic, 23 with probable idiopathic, and 16 with tuberculous, adrenal insufficiency)<sup>1</sup>; all sera were tested for antibodies to gastric parietal cell by complement fixation and immunofluorescence techniques, and were then screened and titrated for antibody to intrinsic factor using radio-immunoassay with charcoal absorption.<sup>2</sup> Tests of gastric function were done on 29 of the patients by the augmented histamine test meal.<sup>3</sup> Schilling tests of vitamin-B<sub>12</sub> absorption<sup>4</sup> were carried out, and the level of vitamin B<sub>12</sub> in the serum was measured.<sup>5</sup> Gastric biopsy, using a Crosby capsule,<sup>6</sup> was done on 18 of the patients. No significant difference in steroid replacement dosage was found in the 3 patient-groups.

Out of the 12 patients with idiopathic Addison's disease, 1 had frank pernicious anæmia (P.A.) and another had latent P.A. Frank P.A. was also established in 1 patient whose Addison's

1. Irvine, W. J., Stewart, A. G., Scarth, L. *Clin. exp. Immun.* 1967, 2, 31.

2. Irvine, W. J. *ibid.* 1966, 1, 99.

3. Kay, A. W. *Br. med. J.* 1953, ii, 77.

4. Schilling, R. F. *J. Lab. clin. Med.* 1953, 42, 860.

5. Girdwood, R. H. *Scott. med. J.* 1960, 5, 10.

6. Crosby, W. H., Kugler, H. W. *Am. J. dig. Dis.* 1957, 2, 236.



GASTRIC FUNCTION IN PRIMARY ADRENAL INSUFFICIENCY

Type of adrenal insufficiency	Gastric-acid secretion in post-histamine hour (mEq. hydrochloric acid)		
	Achlorhydria	>0<5	≥5
Idiopathic .. ..	6*	2	1
Probable idiopathic .. ..	5	4	4
Tuberculous .. ..	..	2	5

\* Including 3 patients with P.A.

disease was probably idiopathic, but P.A. was not recorded in the tuberculous group. All 3 patients with P.A. were diagnosed by the standard methods referred to above; in 1 the intrinsic factor (I.F.) content of the gastric juice was assayed by radio-immunoassay,<sup>7</sup> giving a result of 8 ng. units in the basal hour and 6 ng. units in the post-histamine hour, which is well within the P.A. range. All 3 patients had antibody to I.F. in the serum but only 1 had parietal-cell antibody. 4 other patients in the combined idiopathic and probable idiopathic groups, but none of the patients with tuberculous Addison's disease, had antibody to I.F. in the serum. These 4 all had parietal-cell antibody in the serum and gastric analysis in 2 showed histamine-fast achlorhydria. 3 of the 4 are known to have maintained normal vitamin-B<sub>12</sub> absorption for the period of follow-up (1-3 years) despite the presence of I.F. antibody in the serum.

The incidence of gastric parietal-cell antibody was 50% in the idiopathic group, 35% in the probable idiopathic group, and 6% in the tuberculous group. The incidence of achlorhydria and hypochlorhydria (see accompanying table) was found to be significantly higher in the patients with idiopathic or probable idiopathic adrenal insufficiency than in the tuberculous group. The 3 patients with P.A. are included among the 11 patients in the idiopathic groups shown to have achlorhydria. The 8 patients with hypochlorhydria have normal vitamin B<sub>12</sub> levels in the serum, and in the 4 instances that a Schilling test was done the result was normal. Gastric-biopsy results correlated well with the figures obtained for gastric-acid secretion. The concentration of parietal cells varied inversely with the acid-secretion in response to histamine, and the degree of lymphocytic infiltration was noted to be higher in patients with achlorhydria and hypochlorhydria than in those with normal acid secretion.

Antibodies to adrenal cortex were detected by immunofluorescence and complement fixation. They were detected in the serum of 80% (20/25) of the females with idiopathic or probable idiopathic adrenal insufficiency, and in only 10% (1/10) of the males. No antibody to adrenal cortex was detected in 54 patients with P.A.

Serological evidence of adrenalitis is rare in patients with P.A., but gastritis is common in those with adrenalitis. The association of Addison's disease and Addisonian anemia is being recognised more readily. P.A. has been reported in 7 of a group of 118 patients with Addison's disease<sup>7</sup>; 6 of the 7 were children. As to subclinical disease, atrophic gastritis associated with hypochlorhydria or achlorhydria has been described in Addison's disease.<sup>8</sup> Dr. Meecham and Dr. Wyn Jones suggest that cortisone treatment of their patient explained the apparent ten-year remission in his P.A., perhaps by encouraging some regeneration of gastric mucosa and improved vitamin-B<sub>12</sub> absorption. Our study indicates that, at least in some cases, gastric atrophy with achlorhydria is commonly present many years after steroid replacement dosage has been instituted and maintained in patients with idiopathic adrenal insufficiency. It is also evident that normal vitamin-B<sub>12</sub> absorption can continue in the presence of circulating antibodies to I.F.

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ADRENAL DISEASE AND AUTOIMMUNITY

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**ADRENAL DISEASE AND AUTO-IMMUNITY**

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From an immunological point of view there are two types of human adrenal disorder that are of interest; primary atrophy of the adrenal cortex and adrenal hyperplasia.

*Primary atrophy of the adrenal cortex*

During the past few years I have had the opportunity to study 51 patients with adrenocortical insufficiency (Addison's disease). For scientific purposes the clinical diagnosis of Addison's disease should be substantiated by an ACTH test of adrenal function. In the absence of an adrenal stimulation test, patients with the clinical features of Addison's disease plus non-specific laboratory investigations such as urinary ketosteroid excretion, insulin sensitivity, water tolerance, etc., should be described as having "probable adrenal insufficiency".

Tuberculosis is still a common cause of adrenal failure in middle aged and elderly adults. Other causes include histoplasmosis, syphilis, haemorrhage, infarction, tumour, amyloidosis, etc. During life, the diagnosis of idiopathic adrenal insufficiency (simple atrophy) is made by the process of exclusion and it is sometimes difficult to be certain that one or other of the pathologies just listed may not be responsible.

Radiological evidence of calcification of the adrenal excludes simple atrophy as a cause of adrenal failure. Tuberculosis is the commonest cause of adrenal calcification. If there is evidence of gross tuberculous disease elsewhere in the body prior to or coincident with the Addison's disease, then it is logical to assume a tuberculous cause for the adrenal failure whether or not there is any adrenal calcification on X-ray. On the other hand, a positive Mantoux test or a Gohn focus on chest X-ray would not, at least in Britain, necessarily indicate that the adrenal failure in a patient with Addison's disease was due to tuberculosis and not simple atrophy. Because of these unavoidable uncertainties, I have grouped the patients with Addison's disease into three categories as shown in Table I. The age range and mean age of the patients in the three groups are very similar. The sex ratio in the 35 patients with idiopathic or probable idiopathic Addison's disease is 2.5 female to 1 male; males predominate in the patients with adrenal tuberculosis.

**TABLE I**  
*Classification, age and sex of patients with adrenal insufficiency*

Type	No. of patients	Age in years at diagnosis of adrenal insufficiency		Age in years at time of present study		Sex ratio	
		Range	Mean	Range	Mean	F	M
Idiopathic	11	7-74	38	9-74	44	2.7	1.0
Probable idiopathic	24	12-63	32	17-66	41	2.4	1.0
Tuberculous	16	12-69	38	21-72	49	0.6	1.0

The histology of the adrenal in patients with idiopathic adrenal insufficiency is characterised by reduction in size of the adrenal glands. The normally three-layered architecture of the cortex



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is disrupted. Instead, there are islets of adrenal epithelial cells surrounded by loose fibrous tissue. There is a variable amount of lymphocytic infiltration (Wells, 1930). The adrenal gland of one autopsy that I had the opportunity of studying showed such dense lymphocytic infiltration that the overall size of the adrenals was only slightly less than normal, yet there were very few adrenal epithelial cells to be seen. There was no other pathology noted at the autopsy. Except for the absence of germinal centres, the histology of the adrenal resembled that of the thyroid in Hashimoto's goitre (Irvine, Stewart and Scarth, 1967).

Adrenal specific antibodies have been detected in the serum of a proportion of patients with Addison's disease by complement fixation (Anderson, Goudie, Gray and Timbury, 1957) and by immunofluorescence (Blizzard, Kyle, Chandler and Hung, 1962; Irvine, 1963; Goudie, Anderson, Gray and White, 1966). Figure 1 demonstrates the presence of adrenal antibody in the serum from a patient with idiopathic Addison's disease using the immunofluorescent method. The most intense staining is in the zona glomerulosa. The zona fasciculata and reticularis also stain but less intensely. The adrenal medulla does not stain. Staining occurs only in the cytoplasm of the adrenocortical secretory cells. The antigen is predominantly in the microsomal and to a lesser extent in the mitochondrial fraction of the adrenal cells. The specificity of the antibody has been determined by testing positive sera against liver and kidney and by absorption procedures with thyroid, gastric and adrenal tissues. It is organ-specific but not species-specific. The antibody is an immune globulin of type IgG as determined by the immunofluorescent method using monovalent antisera and by the observation that the adrenal antibody is capable of crossing the placenta into the foetal circulation. An identical titre of the adrenal antibody was detected in the cord blood and in the mother's blood (Irvine, Stewart and Scarth, 1967).



*Fig. 1.* Indirect fluorescent antibody technique using an unfixed frozen section on an adrenal gland from a patient with Cushing's disease. The serum was used in a dilution of 1:4 and was from a patient with idiopathic adrenal insufficiency. The antigen-antibody reaction was detected using horse anti-human immunoglobulin IgG conjugated with fluorescein isothiocyanate (direct ultraviolet light  $\times 250$ ) (reduced 1/3 for reproduction).

The incidence of adrenal antibody in patients with Addison's disease, in controls matched for age and sex and in a range of other conditions, is shown in Table II. With the exception of one patient with tuberculosis of the adrenals whose serum gave a weak titre by complement



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TABLE II

*Incidence of adrenal specific antibodies*

	No. of patients	Antibody positive	
		Fluorescence	Complement fixation
Adrenal insufficiency			
Idiopathic	11	9	6
Probable idiopathic	24	12	10
Tuberculous	16	—	1*
Controls (matched for age and sex)	51	—	—
Thyrotoxicosis	91	—	N.D.
Lymphadenoid goitre	57	1	N.D.
Primary atrophic hypothyroidism	32	—	N.D.
Pernicious anaemia	55	—	N.D.
Diabetes mellitus	56	1	N.D.

\* The antigen in this reaction was localised to capillary and sinusoidal endothelium and not to adrenal secretory cells, using anti  $\beta_1$  A/C conjugate in the indirect immunofluorescent technique. N.D. = not done.

fixation but a negative immunofluorescence test to adrenal secretory cells, positive tests for adrenal antibody only occurred with the sera of patients with idiopathic or probable idiopathic Addison's disease; 80% of the 25 females with idiopathic or probable idiopathic Addison's disease were positive for adrenal antibody, whereas only 1 out of the 10 males in these two groups was positive. The sera of all the 51 control subjects were negative by both tests. Out of 291 sera from patients with thyrotoxicosis, lymphadenoid goitre, primary myxoedema, pernicious anaemia and diabetes mellitus only 2 were positive for adrenal antibody. One was from a patient aged 55 years with lymphadenoid goitre and rheumatoid arthritis. She had a normal plasma level of 17-hydroxycorticosteroids and a normal response to intravenous  $\beta_1$ - $_{24}$  corticotrophin (Wood, Frankland, James and Landon, 1965). The other was from a male diabetic aged 37 years.

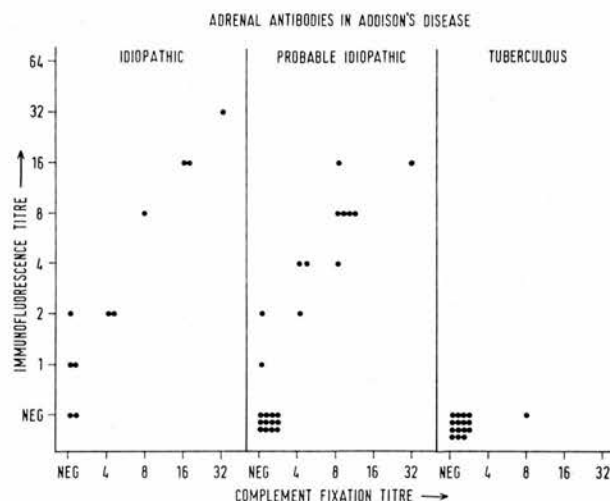


Fig. 2. The titration of adrenal antibodies in the sera of patients with adrenocortical insufficiency using the methods of indirect immunofluorescence and complement fixation. The single positive complement fixation test in the tuberculous group of patients was not directed against adrenal secretory cells but to vascular endothelium.

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Figure 2 shows the titre of adrenal antibody in the three groups of patients with Addison's disease. Along the vertical axis is the titre by immunofluorescence and along the horizontal axis is the titre by complement fixation. There is, with exception of the one tuberculous patient already referred to, a good correlation between the two methods for titrating this antibody. This would suggest that both methods are detecting the same antibody or group of antibodies. The titres of adrenal antibody so far encountered are not high. The range is less than that for parietal cell antibody in patients with pernicious anaemia and certainly less than that for thyroid complement fixing antibody in patients with chronic thyroiditis.

There is a difference between the idiopathic and the tuberculous groups in the incidence of other organ-specific antibodies in the serum. Eight out of 11 patients with idiopathic and 13 out of 24 patients with probable idiopathic Addison's disease had either thyroid or gastric or both types of these antibodies in the serum, while only 3 out of 16 patients with tuberculous Addison's disease had these antibodies. The sera of all but one of the idiopathic group was positive for antibody to thyroid, stomach or adrenal. The sera of 18 out of 24 of the probable idiopathic group contained one or other of these antibodies, but only 3 of the 16 tuberculous patients were similarly positive. The incidence in the tuberculous group was comparable to that in the controls. Although parathyroid antibodies were not studied in the present series, Blizzard, Chee and Davis (1966) reported an incidence of 26% in patients with idiopathic Addison's disease.

TABLE III  
*Immunological survey in adrenal insufficiency*

Type of adrenal disease	No. of patients	Sera positive for antibodies to								A.N.F.	
		Thyroid			Stomach			Thyroid or stomach or adrenal	Thyroid, stomach or adrenal	IgG	IgM
		Tg.	Micr.	Total	P.C.	I.F.	Total				
Idiopathic	11	2	7	7	5	2	6	8	10	—	—
Probable idiopathic	24	4	9	10	9	5	10	13	18	—	1
Tuberculous	16	1	2	2	1	—	1	3	3	—	—
Control subjects (matched for age and sex)	51	N.D.	6		4	—	4			N.D.	N.D.

(N.D. = not done)

Table III shows the type of thyroid and gastric antibodies that occur in the sera of patients with Addison's disease. Thyroid cytoplasmic antibody is more common than is antibody to thyroglobulin. Both types of gastric antibodies occur; antibody to parietal cells and antibody to intrinsic factor. There is a correlation between parietal cell antibody in the serum and simple atrophic gastritis (Irvine, Davies, Teitelbaum, Delamore and Williams, 1965) and a close correlation has been described between antibody to intrinsic factor in the serum and malabsorption of vitamin B<sub>12</sub> due to lack of intrinsic factor (Irvine, 1965; 1966). It is remarkable that 7 of the patients with idiopathic or probable idiopathic Addison's disease should have intrinsic factor antibody. Three of these patients had pernicious anaemia; 3 of the other 4 patients were known to have achlorhydria and all 4 had normal serum levels of vitamin B<sub>12</sub>.

A study was made of gastric function and histology in the different groups of patients with Addison's disease. Table IV summarises the results of the gastric secretion of hydrochloric acid in the posthistamine hour. Achlorhydria is a common finding in patients with idiopathic or probable idiopathic Addison's disease but not in patients with adrenal tuberculosis. Hypochlorhydria occurred in all three groups. Some patients, particularly the tuberculous, had at least a moderate secretion of gastric acid ( $\geq 5$  mEq.HCl).



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TABLE IV

*Gastric function in adrenal insufficiency*

Type of adrenal insufficiency	Gastric acid secretion in post histamine hour (mEq.HCl)		
	Achlorhydria	>0<5	≥5
Idiopathic	6*	2	1
Probable idiopathic	5	4	4
Tuberculous	—	2	5

\* including 2 patients with pernicious anaemia.

(from Irvine, Stewart and Scarth, *Clinical and Experimental Immunology*, 1967. Vol. 2, p. 31)

The gastric histology, studied by Crosby capsule, in 18 of the cases was in keeping with the secretory studies insofar that a reduction in gastric acid secretion was paralleled by a reduction in the number of parietal cells seen in the gastric biopsy. Atrophy was frequently accompanied by an increased degree of lymphocytic infiltration with germinal centre formation. Likewise, lymphocytic infiltration in the thyroid is common in patients with idiopathic Addison's disease but not in patients with adrenal tuberculosis (Schmidt, 1926; Sloper, 1953; Carpenter, Solomon, Silverberg, Bledsoe, Northcutt, Klinenberg, Bennett and McGehee Harvey, 1964).

TABLE V

*Diseases associated with adrenal insufficiency*

	Type of adrenal insufficiency		
	Idiopathic	Probable idiopathic	Tuberculous
Thyroid disease -			
Thyrotoxicosis	1	2	—
Hypothyroidism	1	2	—
Euthyroid goitre	—	1	—
Pernicious anaemia	2	1	—
Hypoparathyroidism	—	3	—
Moniliasis	—	3	—
Diabetes mellitus	2	5	—
Rheumatoid arthritis	—	1	—
Asthma	—	2	—
Eczema	—	1	—
Schilder's disease	1	—	—
Alopecia totalis	—	1	1
Total affected	7	14	1
Total patients	11	24	16

Table V lists the diseases that occurred in the 51 patients with Addison's disease whose serology I have been discussing. It would appear that idiopathic adrenocortical insufficiency is part of a syndrome of polyendocrinopathy, moniliasis and atrophic gastritis. Other disorders possibly associated with aberrant immunological features may also be part of this syndrome.

The predominantly female incidence, the histology of the adrenal atrophy, the detection of organ-specific antibodies in the serum, the overlap clinically and immunologically with other diseases associated with autoimmune phenomena, all point to idiopathic Addison's disease as belonging to the group of diseases that have come to be known as autoimmune.

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*Adrenal hyperplasia*

Wegienka, Wuepper, Komary and Forsham (1966) have reported that, out of 10 patients with Cushing's syndrome, 1 had antibody to adrenal in the serum. These authors also drew attention to the lymphocytic infiltration that is not uncommon in the hyperplastic adrenal gland. It is therefore possible that some cases of adrenal hyperplasia may be analogous immunologically to thyrotoxicosis. To be speculative, there may be an abnormal adrenal stimulating substance, possibly antibody in nature, which may be of aetiological importance in some cases of adrenal hyperplasia.

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CLINICAL AND IMMUNOLOGICAL ASSOCIATIONS  
IN ADRENAL DISORDERS

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### Clinical and Immunological Associations in Adrenal Disorders

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During the past ten years evidence has accumulated that idiopathic adrenal insufficiency belongs to the group of conditions that are associated with organ-specific autoimmunity (Goudie *et al.* 1966, Nerup *et al.* 1966, Blizzard *et al.* 1967, Irvine *et al.* 1967). The present paper summarizes the clinical and immunological associations in 72 patients with primary adrenal insufficiency.

#### Patients studied

The patients with primary adrenal insufficiency were classified into three groups:

*Idiopathic (15):* No adrenal calcification, no history of tuberculosis and no evidence of gross tuberculosis elsewhere in the body. Clinical diagnosis of Addison's disease confirmed by a satisfactory ACTH stimulation test.

*Probably idiopathic (31):* Same as above, but equivocal history of tuberculosis or equivocal minor lesion on chest X-ray. Clinical features typical, but the diagnosis of Addison's disease may not have been confirmed by an adequate ACTH test.

*Tuberculous (26):* Unequivocal evidence of gross tuberculosis with or without calcification of the adrenals. Classical clinical features of primary adrenal failure.

The details of investigation of the first 51 patients with Addison's disease included in the present study have been reported previously (Irvine *et al.* 1967).

#### Methods

The serological procedures used were as described previously (Irvine *et al.* 1967). Tests for adrenal, thyroid and gastric antibodies included immunofluorescence and complement fixation. Antibody to thyroglobulin was detected by tanned cell haemagglutination and by immunofluorescence using fixed sections of human thyroid. Antibody to intrinsic factor was detected by the albumin-coated charcoal method. Antinuclear factor was detected using polyvalent anti-human gamma-globulin in the immunofluorescent procedure

using sections of rat liver; mitochondrial antibodies were detected using sections of rat kidney.

#### Results

The detection of adrenal-specific antibodies by immunofluorescence is illustrated in Fig 1 and the incidence of adrenal-specific antibodies in the 72 patients with primary adrenal insufficiency is shown in Fig 2. The sera of 11 out of 15 patients with primary idiopathic and 14 out of 31 with probable idiopathic Addison's disease were positive for antibody to adrenocortical secretory cells. The antibody is localized to the cytoplasm by the immunofluorescence test and is predominantly in the microsomal fraction by ultracentrifugation and complement fixation analysis. None of the 26 patients with tuberculous Addison's disease had antibody to adrenocortical secretory cells. The one patient in this group with a persistently positive complement fixation test with adrenal tissue showed staining of the vascular endothelium only when tested by immunofluorescence using adrenal sections. In this instance the complement fixation test was probably not reacting with adrenocortical antigen but with a blood group substance.

The age range and mean age of the patients in the three groups was closely comparable. The sex distribution of the patients with Addison's disease is shown in Table 1. In the combined idiopathic groups the female to male ratio is 2.5:1.0, while in the tuberculous group it is 0.9:1.0. In the idiopathic groups 23 out of the 33 females (70%) were positive for adrenocortical antibody whereas only 2 out of the 13 males (15%) were positive.

Sera reacting positively with adrenocortical tissue were tested for specificity with human ovarian tissue, but no reaction was observed. Absorption procedures using thyroid and gastric extracts confirmed the specificity of the antibody for the adrenal gland (Irvine 1963). Tests with liver and kidney sections by immunofluorescence distinguished between mitochondrial antibody and antibody specific for the adrenal.

Table 2 shows the incidence of adrenocortical antibodies in patients with conditions primarily affecting other tissues and which are associated to a greater or lesser extent with the occurrence of autoantibodies. There is only a very low incidence

**Table 1**

Sex incidence of adrenocortical antibodies in primary adrenal insufficiency

Disease group	Sex	Adrenocortical antibodies		Total
		Positive	Negative	
Idiopathic	F	9	1	10
	M	2	3	5
Probable idiopathic	F	14	9	23
	M	0	8	8
Tuberculous	F	0	12	12
	M	0	14	14

of adrenocortical antibodies in these disease states and it is uncertain whether this is statistically different from the 0% incidence in the 72 control subjects so far studied.

Table 3 shows the incidence of other types of organ-specific antibodies in the patients with Addison's disease and in the control subjects matched for age and sex. In the idiopathic groups, there is an increased incidence of thyroid and of gastric antibodies, but the incidence in the tuberculous group is comparable to that in the control subjects. With regard to thyroid antibodies, microsomal antibody was more frequently present than antibody to thyroglobulin. With regard to gastric antibodies, antibody to intrinsic

factor occurred as well as antibody to parietal cell cytoplasm. The patients with intrinsic factor antibody included 3 who had evidence of pernicious anaemia but pernicious anaemia in its frank form had been excluded in the other 5. The vitamin B<sub>12</sub> metabolism in these 5 patients will be followed with particular interest.

In the idiopathic group 63% of the patients had thyroid and/or gastric antibodies in the sera as opposed to 15% of the tuberculous patients.

**Table 2**

Incidence of adrenocortical antibodies in other conditions

	No. of patients	No. of positives
Thyrototoxicosis	89	—
Lymphadenoid goitre	79	3
Primary atrophic hypothyroidism	87	—
Pernicious anaemia	54	—
Diabetes mellitus	93	—
Steatorrhoea	21	1

Adrenal, thyroid and/or gastric antibodies were present in the sera of 76% of the patients in the idiopathic groups as opposed to 15% in the tuberculous patients.

When the patients in whom there is some doubt about a possible tuberculous aetiology or in whom the diagnosis of adrenal failure has not been subject to the confirmation of an adequate ACTH test were excluded, the corresponding figure for the incidence of gastric or thyroid antibodies is 73% and for adrenal, thyroid and/or gastric antibodies is 87% in patients with idiopathic Addison's disease.

*Gastric analysis and histology in patients with Addison's disease:* Gastric analysis using a standard augmented histamine test (0.04 mg body weight subcutaneously) was done in 31

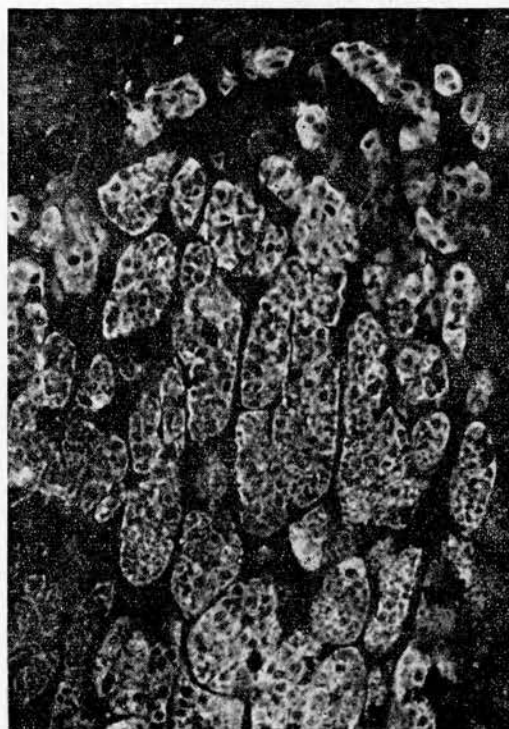


Fig 1 Positive test for antibody to adrenocortical secretory cells in the indirect fluorescent antibody technique using the serum of a patient with idiopathic Addison's disease and anti-IgG conjugate. Dark ground ultraviolet light.  $\times 155$

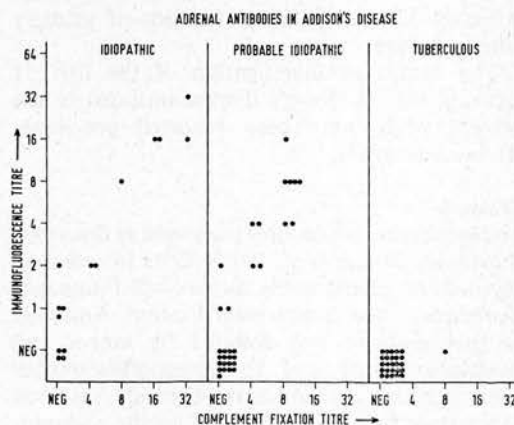


Fig 2 The titration of adrenal antibody in the sera of 72 patients with primary adrenocortical insufficiency using the methods of indirect immunofluorescence and complement fixation



Table 3

Immunological survey in primary adrenal insufficiency

Type of adrenal disease	No. of patients	Sera positive for antibodies to:						Thyroid, stomach or adrenal
		Thyroid			Stomach			
		Tg	Micr	Total	PC	IF	Total	
Idiopathic	15	2	9	9	6	3	7	13
Probable idiopathic	31	5	12	14	12	5	13	22
Tuberculous	26	2	2	3	1	—	1	4
Control subjects (matched for age and sex)	72	4	7	8	6	—	6	11

Tg = thyroglobulin; Micr = microsomal; PC = parietal cell; IF = intrinsic factor; adrenal = adrenocortical secretory cells

patients. In addition, one patient with probable idiopathic Addison's disease had achlorhydria as determined by the tubeless Diagnex test (Table 4). Achlorhydria and hypochlorhydria were a common occurrence in patients with idiopathic or probable idiopathic Addison's disease. Of the 7 patients with tuberculous Addison's disease studied in this respect, 5 had a fair to good gastric acid secretion ( $\geq 5$  mEqHCl in the post-histamine hour), only 2 had hypochlorhydria and none had achlorhydria.

Gastric biopsies were obtained by Crosby capsule in 18 of the patients with adrenal insufficiency. A reduction in the number of parietal cells and of oxyntic cells was noted in patients in whom there was hypochlorhydria or achlorhydria. Gastric atrophy was frequently accompanied, particularly in the idiopathic groups, by an increased degree of lymphocytic infiltration with germinal centre formation.

*Clinical conditions occurring in the patients with Addison's disease:* Overt disease of other tissues occurring in the patients with Addison's disease is listed in Table 5. There is a remarkable incidence of thyroid disorders and of pernicious anaemia, hypoparathyroidism, moniliasis and diabetes mellitus. The patients with diabetes mellitus were insulin dependent; those with hypoparathyroidism and moniliasis were all young subjects. The 2 patients with rheumatoid arthritis were elderly. Three patients suffered from asthma and one gave a history of eczema in childhood. Two of the boys with Addison's disease suffered from neurological conditions; one had the demyelinating condition, Schilder's disease, and another had mental retardation and deafness. A woman aged 28 years with probable idiopathic Addison's disease had idiopathic epilepsy. Alopecia totalis occurred in one patient with tuberculous Addison's disease as well as in one patient with probable idiopathic adrenal failure. With this exception all the above-mentioned conditions were only detected in patients in the idiopathic groups. Indeed, the majority of patients with idiopathic or probable idiopathic Addison's disease suffered from one or more of the conditions listed.

*Incidence of adrenal antibodies in other types of adrenal disorder:* Adrenal antibodies were not detected in any of 15 patients with Cushing's syndrome due to bilateral adrenal hyperplasia as proven by subsequent bilateral adrenalectomy. Adrenal antibodies were not detected in 3 patients with idiopathic hypopituitarism with secondary adrenal failure, but were present in one of 22 patients with hypopituitarism of known aetiology (postpartum haemorrhage, chromophobe adenoma and hypophysectomy):

**Case 1** A woman aged 60 years gave a classical history of postpartum haemorrhage at age 34 years with subsequent failure of pituitary function as manifest by

Table 4

Gastric function in primary adrenal insufficiency

Type of adrenal insufficiency	Gastric acid secretion in post-histamine hour (mEq HCl)		
	Achlorhydria	>0 <5	$\geq 5$
Idiopathic	6●	2	2
Probable idiopathic	7■	4	4
Tuberculous	—	2	5

● Including 3 patients with pernicious anaemia

■ Including one patient with pernicious anaemia in whom achlorhydria was demonstrated by Diagnex test

Table 5

Diseases associated with primary adrenal insufficiency

	Types of adrenal insufficiency		
	Idiopathic	Probable idiopathic	Tuberculous
Thyroid disease:			
Thyrotoxicosis	1	2	—
Hypothyroidism	2	2	—
Euthyroid goitre	—	2	—
Pernicious anaemia	2	2	—
Gastric ulcer	—	1	—
Hypoparathyroidism	1	2	—
Moniliasis	—	3	—
Diabetes mellitus	2	5	—
Rheumatoid arthritis	—	2	—
Asthma	1	2	—
Eczema	—	1	—
Schilder's disease	1	—	—
Mental retardation and deafness	1	—	—
Epilepsy	—	1	—
Alopecia totalis	—	1	1
Total affected	10	16	1
Total patients	15	31	26



failure of lactation, amenorrhœa and loss of body hair. Absence of growth hormone secretion was demonstrated following stimulation by insulin hypoglycæmia (Dr W M Hunter). Thyroid deficiency was demonstrated by radioactive iodine studies but no increase in  $^{131}\text{I}$  uptake by the thyroid could be induced by repeated TSH stimulation. Likewise, only a minimal increase in the urinary excretion of 11-OHCS could be produced by 5 days' stimulation with ACTH gel 40 units twice a day. This patient therefore appeared to have pituitary failure secondary to post-partum hæmorrhage, but also to have primary thyroid and primary adrenal insufficiency. Her serum was positive for antibody to adrenocortical secretory cells by immunofluorescence and by complement fixation (titre=1:8).

In none of the patients with hypopituitarism could antibodies reacting with human pituitary tissue in the immunofluorescence test be detected.

#### Discussion

The pronounced difference in the incidence of adrenal antibodies in patients with idiopathic Addison's disease contrasts with the low incidence in patients with tuberculous destruction of the adrenal. This is important to the clinician in that its finding indicates a diagnosis of autoimmune adrenalitis and would tend to exclude tuberculosis and possibly other causes of adrenal failure such as hæmorrhage, carcinomatosis, amyloidosis, &c. Patients with idiopathic Addison's disease have a high incidence of other clinical and subclinical conditions, particularly associated with autoimmunity. The gastric function and histology studies reported here demonstrate a high incidence of subclinical atrophic gastritis, in addition to those patients who have pernicious anæmia as well as adrenal failure. Carpenter *et al.* (1964) have reported an increased incidence of thyroiditis in autopsy studies on patients with idiopathic Addison's disease. This is in keeping with the increased incidence of thyroid antibodies noted in the present study in the idiopathic groups.

The fact that adrenal antibodies occur only in idiopathic Addison's disease and not when it is due to tuberculosis indicates that these antibodies are not simply the consequence of adrenal damage. This observation supports the concept that the group of organ-specific autoimmune diseases, which include thyroid, stomach, adrenal and parathyroid, are associated with a disorder of immunological tolerance (Irvine 1964).

The preponderance of females to males in idiopathic Addison's is in contrast to the apparently equal sex ratio in tuberculous Addison's disease and is in keeping with the former being an autoimmune condition. The higher incidence of adrenal antibodies in the female subjects than in the male subjects with idiopathic Addison's

disease is difficult to explain as a similar difference in the incidence of antibodies according to sex is not observed in relation to other conditions associated with organ-specific autoimmunity.

The low incidence of adrenal antibodies in patients with other types of organ-specific autoimmune disease correlates with the comparative rarity of idiopathic Addison's disease and with the observation that focal adrenalitis is not a common histological finding at routine autopsies.

The finding of adrenal antibodies in high titre in one patient with steatorrhœa lends some support to the observation by Kunin *et al.* (1963) that steatorrhœa may be associated in some way with adrenal insufficiency, particularly in childhood. The association of insulin-dependent diabetes mellitus with the group of organ-specific autoimmune diseases is emphasized. Further attention should be paid to the possible association between neurological disorders and idiopathic adrenal failure, particularly in children.

Although one case is reported in the present paper of pituitary failure associated with primary failure of thyroid and adrenal glands, there is as yet no direct evidence for an autoimmune basis for idiopathic pituitary failure. A degree of lymphocytic infiltration of the pituitary has been observed in patients with primary atrophy of the adrenal (Susman 1930, Carpenter *et al.* 1964, Irvine *et al.* 1967). It is therefore conceivable that an autoimmune hypophysitis might exist.

There is no evidence in the present study that bilateral adrenal hyperplasia may have an autoimmune basis in a manner comparable to thyrotoxicosis as suggested by Wegienka *et al.* (1966).

It is suggested that the term 'idiopathic adrenal failure' be replaced by the term 'chronic autoimmune adrenalitis'.

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IMMUNOLOGICAL ASPECTS OF PREMATURE  
OVARIAN FAILURE ASSOCIATED WITH  
IDIOPATHIC ADDISON'S DISEASE

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# IMMUNOLOGICAL ASPECTS OF PREMATURE OVARIAN FAILURE ASSOCIATED WITH IDIOPATHIC ADDISON'S DISEASE

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**Summary** Antibodies reactive with the theca interna of the ovary, the interstitial cells of the testis, and the adrenal cortex were detected in the sera of 5 of 77 patients with idiopathic adrenal insufficiency. All 5 of these patients had either complete failure of menstruation or an early menopause. 1 of these 5 patients also had antibody reactive with ova and spermatids, and 3 had antibody reactive with placental trophoblasts. Only 1 of the remaining 72 patients with idiopathic Addison's disease was known to have premature failure of menstruation. Antibody reactive with gonadal tissue was absent in 34 patients with tuberculous Addison's disease, 111 control subjects matched with the Addison's patients for

age and sex, 43 patients with gonadal dysgenesis that was not associated with Addison's disease, 50 postmenopausal females, and in 251 subjects with pernicious anæmia or thyroid autoimmune disease. Antibody reactive only with the theca interna of human ovary and with adrenal cortex was found in 1 of 24 patients with Cushing's syndrome. Primary gonadal failure may be part of a polyendocrinopathy of which idiopathic Addison's disease is an integral part and which is characterised by the formation of autoantibodies to the respective tissues.

#### Introduction

A HIGH incidence of organ-specific autoantibodies in the sera of patients with idiopathic Addison's disease is now well recognised (Goudie et al. 1966, Nerup et al. 1966, Blizzard et al. 1967, Irvine, Stewart, and Scarth 1967), and Turkington and Lebovitz (1967) have described premature ovarian failure in Addison's disease. We have attempted to determine if antibodies reactive with ovarian constituents are a feature of Addison's disease and, if so, how the presence of these antibodies correlates with the patient's gonadal function.

#### Patients and Methods

##### *Patients*

111 patients with Addison's disease were investigated serologically. The patients were subdivided into those with idiopathic and those with tuberculous adrenal failure and were matched for age and sex with blood-donors. 324 patients with gonadal dysgenesis, addisonian pernicious anæmia, autoimmune thyroid disease, or Cushing's syndrome, and a further 50 postmenopausal female controls aged fifty to fifty-nine were also included (table 1).

##### *Methods*

Each serum was tested against human adrenal gland, ovary, testis, and placenta and also against rabbit ovary and testis. Ovarian tissue was obtained by biopsy from 2 patients aged forty-two and forty-four undergoing surgery for uterine fibroids, and the testicular tissue was taken from 2 patients aged sixty-one and sixty-two at orchidectomy for prostatic carcinoma. Adrenal tissue was obtained from patients undergoing bilateral adrenalectomy for diffuse metastatic carcinoma. Ovaries from rabbits of a reproductive age at various stages of the oestrous cycle were used since they contained a larger number of ova and luteal cells than the human ovary tissue that was available. Testicular tissue from young adult rabbits was also used.



Sera were tested against these tissues using the indirect immunofluorescence method with antihuman IgG conjugated with fluorescein isothiocyanate as previously described (Irvine, Stewart, and Scarth 1967). The intensity of the staining was assessed subjectively as ++, +, or -. The immunoglobulin specificity of positive reactions with steroid-producing tissues was checked using antihuman IgA and antihuman IgM conjugates. The tissue specificity was determined using sections of human thyroid and gastric mucosa and rat kidney. The sera of the patients with adrenal disorders were also tested for

TABLE I—INCIDENCE OF ANTIBODIES TO ADRENAL AND TO OTHER STEROID-PRODUCING CELLS

Disease	No. of patients	Sera positive for antibodies to:	
		Human adrenal cortex	Other steroid-producing cells in human tissue
Idiopathic adrenal insufficiency ..	77	40	5
Tuberculous adrenal insufficiency..	34	0	0
Controls (matched for age and sex)	111	0	0
Gonadal dysgenesis .. ..	49	0	0
Pernicious anaemia .. ..	102	0	0
Lymphadenoid goitre .. ..	49	0	0
Primary hypothyroidism .. ..	50	0	0
Thyrotoxicosis .. ..	50	0	0
Cushing's syndrome .. ..	24	1	1
Post-menopausal females (aged 50-59) .. ..	50	0	0

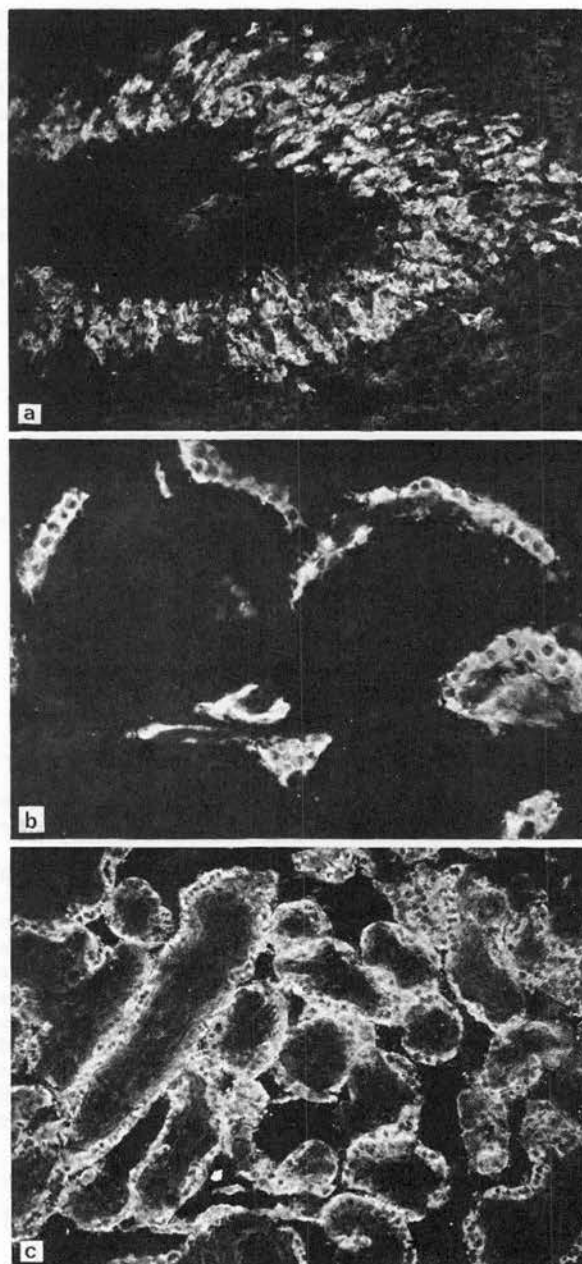
antibody to thyroglobulin by the tanned-cell hæmagglutination method (Fulthorpe et al. 1961) and for intrinsic-factor antibody I (Irvine 1966).

Sera giving positive immunofluorescence reactions with steroid-producing tissues were titrated by the indirect immunofluorescence method using tissue sections and by complement fixation using saline extracts of the various tissues. The immunofluorescence method was also used to determine the titre of antibody reacting with steroid-producing tissues before and after the sera were absorbed with buffered saline extracts of human adrenal, testis, placenta, thyroid, and stomach and of rat liver. The saline tissue extracts were prepared as previously described (Irvine, Stewart, and Scarth 1967). Equal volumes of serum and extract were mixed at room temperature for three hours.

### Results

#### Serology

The incidence of positive immunofluorescence tests



Immunofluorescent staining (indirect method using antihuman IgG conjugated with fluorescein isothiocyanate, and dark-ground ultraviolet microscopy):

(a) Graafian follicle of human ovary with the serum of patient E (reduced to  $\frac{1}{2}$  of  $\times 260$ ); (b) interstitial cells of human testis with the serum of patient E (reduced to  $\frac{1}{2}$  of  $\times 375$ ); and (c) human placental trophoblasts with the serum of patient A (reduced to  $\frac{1}{2}$  of  $\times 260$ ).

TABLE II.—POSITIVE IMMUNOFLOUORESCENT REACTIONS FOR ANTIBODY TO OVARY, TESTIS, OR PLACENTA CORRELATED WITH CLINICAL FEATURES

Patient	Sex	Age	Main clinical features (and age of onset or diagnosis)	Sera positive for antibody to						
				Adrenal cortex (human)	Theca interna (rabbit and human)	Interstitial cells (rabbit)	Ova (rabbit)	Interstitial cells (rabbit and human)	Sperms (rabbit)	Placenta
A	F	17	Idiopathic adrenal insufficiency (12 $\frac{1}{2}$ ) Idiopathic hypoparathyroidism (1 $\frac{1}{2}$ ) Small goitre (12 $\frac{1}{2}$ ) No menarche (primary amenorrhoea)	++	++	++	++	++	+	++
B	F	20	Idiopathic adrenal insufficiency (15) Primary atrophic hypothyroidism (15) Idiopathic hypoparathyroidism (16) Pernicious anaemia (20) Moniliasis (7) Alopecia totalis (7) No menarche (primary amenorrhoea)	++	++	++	-	++	-	+
C	F	35	Idiopathic adrenal insufficiency (32) Normal menarche (amenorrhoea since age 27)	+	+	+	-	+	-	+
D	F	34	Idiopathic adrenal insufficiency (23) Normal menarche (14) Normal pregnancy (21) Amenorrhoea from age of 26	++	+	+	-	+	-	-
E	F	26	Idiopathic adrenal insufficiency (13) Monilial infection of nails (19) Alopecia areata (19) Normal menarche (14) Amenorrhoea from age of 18	++	++	++	-	++	-	-
F	F	43	Cushing's syndrome with adrenal adenoma (42) Diabetes mellitus (39) Menorrhagia (39)	+	+	-	-	-	-	-

\* Human tissue only.

with adrenal and with other steroid-producing tissues in the 596 patients studied is summarised in table I. Sera reactive with any of the steroid-producing tissues were confined to those patients with idiopathic Addison's disease, with the exception of 1 of 24 patients with Cushing's syndrome that reacted with adrenal cortex and human (but not rabbit) ovary and 1 of 50 thyrotoxic patients that gave a weak reaction with rabbit ovary but not with human steroid-producing tissues. 5 of the total of 41 sera reacting with human adrenal cortex also reacted with ovary, testis, or placenta (table II). Positive immunofluorescence tests with theca interna and adjacent layers of the follicular epithelium of human ovary, with the interstitial cells of the rabbit testis, and with human placental trophoblasts are shown in the figure.

The serum of patient A (table II) gave a strongly positive immunofluorescence test with all steroid-producing cells (human or rabbit) and also reacted with ova and spermatids. The sera of patients B and C reacted with adrenal cortex, theca interna, interstitial cells of ovary and testis, and with placental trophoblasts, but did not react with germ cells. The sera from patients D and E reacted with adrenal cortex, theca interna of the ovary, and interstitial cells of the ovary and testis, but did not react with placenta or germ cells. The serum of patient F (Cushing's syndrome) reacted with adrenal cortex and gave a positive immunofluorescence test with theca interna of human ovary, but not with rabbit ovary nor with the interstitial cells of testis.

The complement-fixation titres for antibody to human adrenal, testis, and placenta are shown in table III. Where there was a reaction with more than one steroid-producing tissue (i.e., all except patient F), the complement-fixation titres were generally of the same order. This was also true

TABLE III—COMPLEMENT-FIXATION TITRES FOR ANTIBODY TO STEROID-PRODUCING TISSUES (HUMAN) IN THE SERA OF PATIENTS INCLUDED IN TABLE II

Patient	Adrenal extract at dilution 1/16	Testis extract at dilution 1/8	Placenta extract at dilution 1/16
A	32	16	32
B	8	16	8
C	4	8	8
D	16	8	—
E	64	64	—
F	32	—	—





when the titres were determined by the indirect immunofluorescence method. The antibodies reactive with steroid-producing tissues were all IgG, no fluorescence being obtained with anti-IgA conjugates and only occasional weak fluorescence when anti-IgM conjugates was used.

When the sera were absorbed with a saline extract of human adrenal, the immunofluorescence titres were greatly reduced or abolished not only with regard to adrenal reactivity but also to ovary, testis, and placenta. Likewise, with regard to patients A-E (table II), absorption with human testis extract not only prevented the immunofluorescence reaction with testicular tissue but also reduced or abolished the reaction between the serum and adrenal cortex, theca interna of the ovary, and placenta. Absorption with testicular extracts had no effect on the immunofluorescence reaction with ovary or adrenal given by serum from patient F, or adrenal-antibody titres in the sera of 2 patients who only had adrenal antibody and whose serum did not react with other steroid-producing tissues. Rabbit ovary was only weakly effective in absorbing reactivity with human ovarian tissue. There was insufficient human ovarian tissue for absorption studies. Absorption with saline extracts of human thyroid, human gastric mucosa, and rat liver had no effect on the titres of antibody to steroid-producing tissue.

The complement-fixation chessboard titrations with the sera in table II and adrenal extract showed two patterns. Sera A, B, and C gave a biphasic pattern as shown for serum B in table IV, while sera D, E, and F gave a monophasic pattern as shown for serum D. All sera reactive with testis gave a similar complement-fixation chessboard titration with testis extract.

The incidence of antibodies to thyroid and stomach and the absence of antinuclear factor and of mitochondrial antibodies in the sera of patients included in table II are shown in table V.

#### *Clinical Findings*

The main clinical findings of the 6 patients with antibodies reactive with steroid-producing tissues in addition to adrenal are summarised in table II. Patients A and B had primary amenorrhoea with no menarche. These patients had the highest incidence of associated autoimmune disorders (idiopathic hypoparathyroidism, idiopathic hypothyroidism, and Addisonian pernicious anaemia)

TABLE V—OTHER SEROLOGICAL FINDINGS IN PATIENTS INCLUDED IN TABLE II

Patient	Thyroid antibodies		Gastric antibodies		Anti-nuclear factor	Mitochondrial antibody
	Thyroglobulin	Microsomal	Parietal cell	Intrinsic factor		
A	—	—	++	+	—	—
B	+	++	—	++	—	—
C	—	—	++	—	—	—
D	—	—	—	—	—	—
E	—	—	—	—	—	—
F	—	—	—	—	—	—

besides their Addison's disease. The onset of adrenal failure in these 2 patients was either before or about the time that puberty would be expected. Patients C, D, and E had a normal menarche, persistent amenorrhœa developing four to fourteen years later but long before the menopause would be expected. In patients D and E, adrenal failure was diagnosed before the onset of the amenorrhœa, but in patient C the amenorrhœa preceded the diagnosis of adrenal failure by five years. All these 5 patients were on adequate replacement therapy for adrenocortical failure and for their other conditions. Despite this, menstruation did not return, except when cyclical œstrogen therapy was used. A urinary total gonadotrophin excretion of >48 mouse units in twenty-four hours was demonstrated on at least one occasion by the mouse-uterus test in patients A, C, D, and E.

Patients A and B were the only ones to be below the third percentile in height. Patient A had bilateral coxa vara and bilateral shortening of the fourth metacarpal. At laparotomy her ovaries were vestigial streaks and on biopsy no follicles or tubules were seen in the region of either ovary. The specimens consisted predominantly of fibrous tissue, and no lymphocytic reaction was noted. The chromosome constitution of patients A-E, as determined by lymphocyte culture, was normal.

Patient F, with Cushing's syndrome, gave a history of menorrhagia rather than amenorrhœa. The menstrual history of the thyrotoxic patient whose serum reacted with the theca interna of rabbit (but not human) ovary and which did not react with other steroid-producing tissues was not remarkable. The steroid-cell-antibody pattern in these 2 patients is more limited than in any of the 5 patients with persistent amenorrhœa.

Only 1 of the remaining patients with idiopathic or tuberculous Addison's disease (whose sera were negative for ovary, testis, or placenta) was known to have persistent amenorrhœa at a young age. She was a twenty-six-year-old girl with idiopathic Addison's disease and idiopathic hypoparathyroidism diagnosed at age twenty-three and twelve, respectively. Menarche happened at age eleven and her periods were regular until age thirteen. At age fourteen, her menstruation ceased and has not recurred.

#### Discussion

Extra-adrenal endocrine disorders in patients with idiopathic Addison's disease have been well documented, particularly with reference to thyroid diseases, diabetes mellitus, addisonian pernicious anæmia, and idiopathic hypoparathyroidism (Beaven et al. 1959, Carpenter et al. 1964, Irvine 1968). The demonstration of a high incidence of organ-specific antibodies in this group of patients has led to the hypothesis that these conditions may to some extent have a common pathogenesis in which autoimmunity may play a relevant part (Blizzard et al. 1967, Irvine, Stewart, and Scarth 1967).

Turkington and Lebovitz (1967) reported in a separate series that of 32 patients with idiopathic adrenal insufficiency 7 had primary gonadal failure: 3 had atrophic testes, 3 had premature menopause, and 1 had cystic ovaries. Premature ovarian failure preceded the development of clinical Addison's disease by five to fourteen years in all of their 3 patients with premature menopause. In our series, adrenal failure preceded ovarian failure in 2 of the 3 patients who had premature menopause after a normal menarche. There seems to be no set sequence for gonadal and adrenal failure. The association between gonadal failure and adrenal failure of the idiopathic (as opposed to the tuberculous) form accords with the other endocrine disorders associated with Addison's disease (Irvine 1968). In their own series and in a review of earlier cases, Turkington and Lebovitz (1967) noted that primary hypothyroidism was a common additional feature in patients with gonadal and adrenal failure. We have been impressed with the frequency of idiopathic hypoparathyroidism and have noted addisonian pernicious anæmia as well as primary atrophic hypothyroidism.

Antibodies reactive with the follicular epithelial cells



and theca interna of the graafian follicles of the ovary and with the interstitial cells of ovary and testis have not previously been described in patients with premature gonadal failure. Christy et al. (1962) described primary failure of ovary, thyroid, and adrenocortical function in a woman of forty, and later reported the presence of thyroid and adrenal antibodies in her serum, but antibody to ovary could not be detected by immunofluorescence tests (Blizzard et al. 1963). Vallotton and Forbes (1966) described antibodies to the cytoplasm of rabbit ova in 29 of 232 sera tested. In 9 of the 29 menstruation had ceased precociously at age thirty-nine or earlier. These workers made no mention of antibodies to other components of ovary or to testis, adrenal, or placenta. Although, in our series, one patient had antibody reactive with ova and spermatids, this serum also reacted with theca interna of ovary and the interstitial cells of ovary and of testis as well as with adrenal cortex and placental trophoblasts. We have been unable to confirm the findings of Vallotton and Forbes (1966, 1967) of antibodies to ova in patients with gonadal dysgenesis associated with chromosome abnormalities (Price and Irvine 1968) in control patients or in patients in whom a high incidence of organ-specific autoantibodies would be expected. Anderson et al. (1968) reported 2 out of 40 patients with idiopathic adrenal insufficiency with antibodies to adrenocortical cells, interstitial and hilus cells of ovary and of testis, cells of the theca interna of the graafian follicle, and luteal cells of the ovary as well as with placental trophoblasts. 1 of these patients was a twenty-two-year-old male with associated steatorrhœa and idiopathic hypoparathyroidism but no obvious evidence of gonadal failure. The case-history of the other patient was not mentioned. One of the most striking features in our series was the strong correlation between premature ovarian failure and the presence in the serum of antibodies reactive with ovary and testis as well as adrenal.

The pattern of immunofluorescence staining given by the various steroid-producing tissues and the results of the absorption studies indicate that there are antigens shared between the adrenal cortex, theca interna of the ovary, and the interstitial cells of the ovary and testis and the placental trophoblasts. From the variation in the immunofluorescent reactions given by different sera, there would seem to be more than one such antigen, but that all of

these antigens are present in the adrenal cortex. This would accord with the fact that both oestrogen and testosterone synthesis happen in the adrenal cortex, and with the observation that the different sera reactive with various steroid-producing tissues gave more than one pattern in complement fixation chessboard titration with the same adrenal extract. The antigens are probably related to enzymes involved in various metabolic pathways of steroid synthesis: the antigen may or may not be a constituent of more than one steroid-producing tissue, depending on the nature of the enzyme.

If antigens are indeed shared between the gonads and the adrenal cortex, one might expect to see ovarian and testicular lesions after immunisation of animals with adrenal extracts in Freund's adjuvant. We are not aware of any histological work on the ovaries of women with premature menopause but, with the advent of the laparoscope, this should not be long in forthcoming.

#### Addendum

The sera of the 111 patients with Addison's disease, the matched controls, the 24 patients with Cushing's disease, the 49 patients with gonadal dysgenesis, and 14 females who were doubly chromatin positive were tested by the indirect immunofluorescent method against human corpus luteum obtained at biopsy from a patient aged thirty-two at termination of a three-month pregnancy. The sera of patients A-F (table II) gave a positive reaction. Sera A, B, and C gave a clumpy staining diffusely throughout the corpus luteum, serum E a patchy staining, and sera D and F a confluent pattern of immunofluorescent staining. Sera A-E also gave a bright immunofluorescent staining of a layer of cells immediately adjacent to the periphery of the corpus luteum and in its trabeculae. These cells were considered to be those of the theca interna. The sera of 2 other patients with idiopathic Addison's disease gave positive tests with this human ovary. One gave a weak granular, membranous staining of the corpus luteum but did not react with other steroid-producing cells in human or rabbit tissue, while the other reacted only with the theca-interna cells adjacent to the corpus luteum and with human adrenal cortex. Neither of these 2 patients had amenorrhoea. The remaining 301 sera tested were negative for antibody to corpus luteum or the surrounding theca interna.

Absorption studies with human adrenal and with human testis extracts showed that the diffuse clumpy staining of the corpus luteum given by sera A and C could be absorbed by human adrenal extract or by human testis extract but that the patchy immunofluorescent staining given by serum E and the confluent staining given by serum F could only be absorbed by human adrenal extract and not by testis extract. Likewise, absorption with either human adrenal or human testis could prevent the reaction with the theca interna given by sera A and C, but only adrenal extract was effective with sera D and E. These findings confirm that there is more than one antibody involved in the reaction with steroid-producing cells, but that all the corresponding antigens are represented in the adrenal cortex.

Since she was anxious to have full investigation with regard to the prognosis in relation to her fertility, laparoscopy with ovarian biopsy was performed on patient E (table II). The ovaries were of normal size. Biopsy showed the presence of numerous primordial follicles. As the follicles enlarged, there was a progressively severe lymphocytic and plasma-cell infiltration with destruction of the follicle. Immunofluorescent studies with the patient's own serum and with serum A showed that the membrana granulosa of the early follicles is poor in antigen—reacting very weakly with serum A and not at all with her own serum. The ova in these follicles reacted strongly with serum A but not with serum B–F (i.e., including her own serum). There was, therefore, no evidence of any immunological attack against her own primordial follicles. When these follicles attempted to develop under the influence of her elevated gonadotrophin levels, the follicular epithelium became antigenic and showed immunofluorescence staining with her own serum. This was associated with lymphocytic and plasma-cell infiltration in these follicles. The destruction of the follicles was such as to preclude normal ovarian function. One might speculate that normal ovarian function might be restored in this patient by the use of immunosuppressant doses of steroids.

While the ovarian microscopical appearance in patient A was that of dysgenesis or agenesis, no chromosome abnormality was detectable. Since the patient had antibodies against all ovarian constituents, including ova, the possibility exists that the destruction of this patient's



ovaries may have occurred in early life as a result of an immunological process.

In conclusion, there is serological and histological evidence that some cases of premature ovarian failure may have an autoimmune basis. In such cases, there is a strong clinical association with idiopathic adrenal insufficiency. Immunologically this is explained by the sharing of antigens between the adrenal cortex and the ovary.

We thank Prof. Russell Fraser for permission to report details of patients C and E, and Miss E. Burton (Department of Gynaecology, Hammersmith Hospital) for performing the laparoscopy and ovarian biopsy on patient E. M. M. W. C. is in receipt of a Commonwealth Scholarship. W. J. I. would like to thank the many physicians in different countries who have kindly sent him sera from patients with Addison's disease.

Requests for reprints should be addressed to W. J. I.

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The further characterization of autoantibodies reactive with  
extra-adrenal steroid-producing cells in patients with adrenal  
disorders

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## THE FURTHER CHARACTERIZATION OF AUTOANTIBODIES REACTIVE WITH EXTRA-ADRENAL STEROID-PRODUCING CELLS IN PATIENTS WITH ADRENAL DISORDERS

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### SUMMARY

Antibodies reactive with the steroid-producing cells in the gonads are described in the sera of ten patients, the majority of whom were known to have idiopathic adrenal insufficiency (Addison's disease) associated with premature ovarian failure. The immunofluorescent staining pattern of these antibodies with steroid-producing cells in the ovary, testis, placenta and adrenal cortex are illustrated. The staining patterns and the results of absorption studies indicate that there are a multiplicity of antibodies reacting with different antigens in the ovary and to a lesser extent in the testis. Most of these antigens are also represented in the adrenal cortex, but are not evenly distributed throughout the cortex. Some of these antigens are not represented in the zona glomerulosa while others are not represented in the zona reticularis.

### INTRODUCTION

Antibodies to steroid-producing cells in the gonads and placenta, cross-reacting with adrenal cortex, have been described in the sera of two patients by Anderson *et al.* (1968) and in the sera of six patients by Irvine *et al.* (1968). The latter observed a good correlation between the presence of these antibodies in the sera reactive with steroid-producing cells in the ovary and a clinical history of premature or complete failure of ovarian function. Five of the six patients had idiopathic adrenal insufficiency (Addison's disease) and one had Cushing's disease. The present paper describes in greater detail some of the characteristics of antibodies reactive with steroid-producing cells.

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## MATERIALS AND METHODS

Ten sera (A-J) reacting with ovary, testis or placenta were included in the present study. Sera A-F have previously been referred to (Irvine *et al.*, 1968). Patients A-E had idiopathic Addison's disease with premature or complete ovarian failure. Patient F had Cushing's disease. Patient G was a 72-year-old female with idiopathic Addison's disease, pernicious anaemia, diabetes mellitus and idiopathic hypothyroidism. Patient H was a 16-year-old male who developed idiopathic Addison's disease at the age of 15 years and who was not known to have any associated endocrinopathy although his pubic hair was noted to be sparse. Patient I was a female aged 51 years with idiopathic Addison's disease and was not known to have any other endocrinopathy. Patient J was a 20-year-old girl with primary hypoparathyroidism at 2 years, idiopathic Addison's disease at 19 years and who had normal menstruation (Irvine, 1969; Irvine & Scarth, 1969).

The incidence of antibodies reactive with steroid-producing cells of the gonads in the sera of patients with Addison's disease has been indicated previously and also the rarity of this group of antibodies in the sera of patients with other diseases characterized by organ-specific autoimmunity and in the sera of control subjects (Irvine *et al.*, 1968).

The procedure used in the indirect immunofluorescent antibody technique was as described by Irvine, Chan & Williamson (1969) using horse anti-human IgG-FITC conjugate with a fluorescein-protein ratio of 0.67. The human tissues used in the fluorescent antibody technique were obtained as follows: adrenal from patients undergoing bilateral adrenalectomy for metastatic carcinoma, testis from a 61-year-old male undergoing orchidectomy for prostatic carcinoma, biopsy specimens from the ovary of a 42-year-old woman undergoing surgery for uterine fibroids and from the corpus luteum of a 32-year-old woman at termination of pregnancy at 12-14 weeks. The human placenta was obtained at a normal full term delivery. Ovaries were obtained from rabbits in oestrus and in pseudopregnancy. Rabbit testis was also used. All sera were also tested against rat kidney sections as controls to exclude the presence of 'M' antibodies (Doniach *et al.*, 1966).

Absorption studies on selected sera were done with veronal buffer extracts of human adrenal, human testis, rabbit ovary and human placenta and with serum E using extracts of human thyrotoxic thyroid tissue, mucosa of the body of human stomach and rat liver. Extracts of these tissues were prepared by homogenizing the tissue with 3 volumes of veronal buffer, centrifuging at 1750 g for 10 min. Absorption was done by incubating the supernatant with 1 volume of serum with continuous mixing for 3½ hr at room temperature, followed by repeat centrifugation.

## RESULTS

The results with sera A-J using adrenal, ovary, testis and placenta in the indirect immunofluorescent antibody test are summarized in Table 1.

*Ovary*

In the rabbit ovary it was noted that certain sera gave immunofluorescent staining of the theca interna and also of the outer layer of granulosa cells of the larger Graafian follicles (Figs. 1, 2 and 3). Whether or not the outer layer of the granulosa cells was stained as well as the theca cells seemed to depend upon the stage of development of the Graafian follicles. This aspect was not studied in detail. Staining of the granulosa and theca cells of human

TABLE 1. Results in the indirect immunofluorescent antibody technique with sera A-J tested against the various steroid-producing tissues

Patient	Sex	Age	Human ovary			Rabbit ovary		Testis		Placenta		Adrenal cortex	
			Corpus luteum			Granulosa cells/theca	Hyperplastic interstitial cells	Human	Rabbit	Human		Glomerulosa	Fasciculated
			Clumpy	Confluent	Patchy			Interstitial	Spermatids				Reticularis
A	F	17	++	++	neg	++	++	++	++	++	++	++	+
C	F	35	++	++	neg	++	neg	neg	neg	++	++	++	++
J	F	20	++	++	neg	++	++	nt	++	++	++	++	++
B	F	20	neg	++	+	++	neg	++	neg	+	++	++	++
E	F	43	neg	++	++	++	neg	++	neg	neg	++	++	++
H	M	16	neg	++	++	++	neg	++	neg	neg	++	++	++
D	F	34	neg	++	neg	++	neg	neg	neg	neg	++	++	++
F	F	43	neg	++	neg	++	neg	neg	neg	neg	++	++	++
G	F	72	neg	++	neg	++	++	neg	neg	++	++	++	neg
I	F	51	neg	++	neg	++	neg	nt	neg	neg	neg	++	+

nt = Not tested.

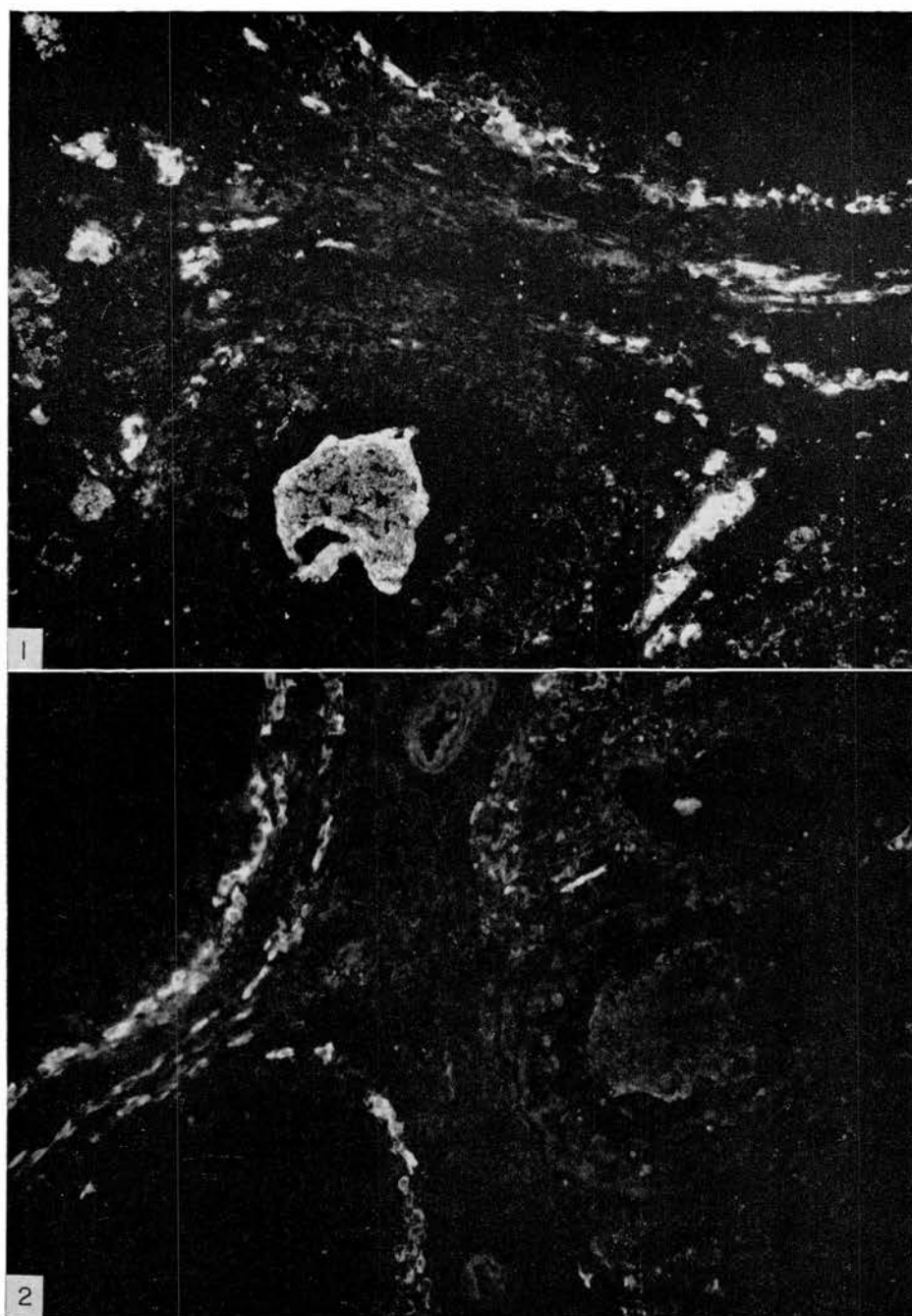


FIG. 1. Positive reaction given by serum A in the indirect immunofluorescent method with the outer layer of granulosa cells and with the theca cells of a fairly large Graafian follicle, with scattered interstitial cells, and with ovum. There was little or no reaction with the granulosa cells of the small follicle containing the ovum. Dark ground UV-blue light,  $\times 225$ .

FIG. 2. Positive immunofluorescence with a layer of granulosa cells and a layer of theca cells in relation to a fairly large Graafian follicle in rabbit ovary using serum E, but negative reaction interstitial cells and with ovum. Dark ground UV-blue light,  $\times 230$ .

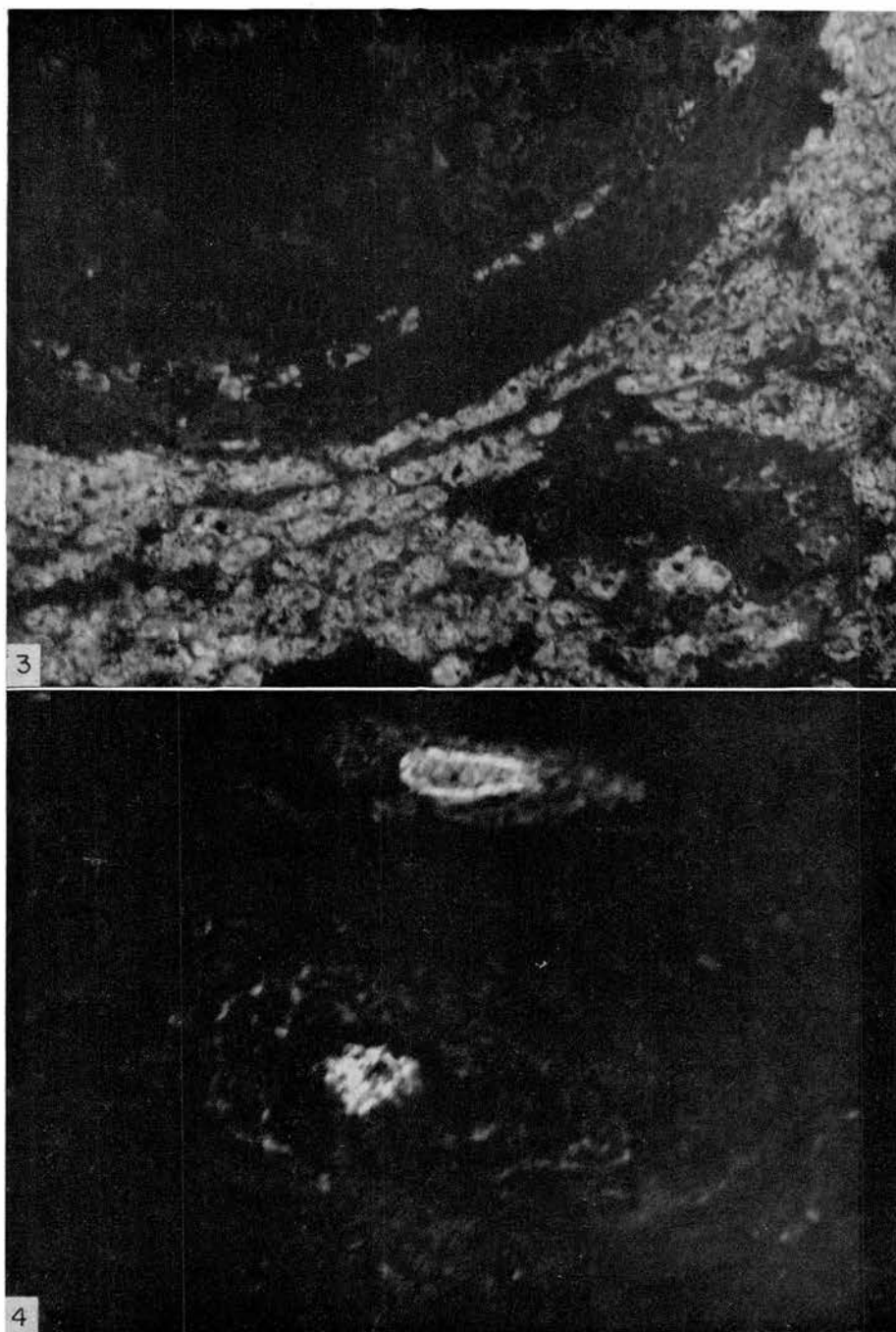


FIG. 3. Positive immunofluorescence given by serum A with a layer of granulosa cells in relation to a fairly large Graafian follicle, and with the hyperplastic interstitial cells characteristic of the rabbit ovary in pregnancy or pseudopregnancy. Dark ground UV-blue light,  $\times 280$ .

FIG. 4. Positive immunofluorescence with ova and weakly with the granulosa cells of the primordial or early follicles in the ovary of patient E using the serum of patient A in the indirect fluorescent antibody technique. Dark ground UV-blue light,  $\times 360$ .



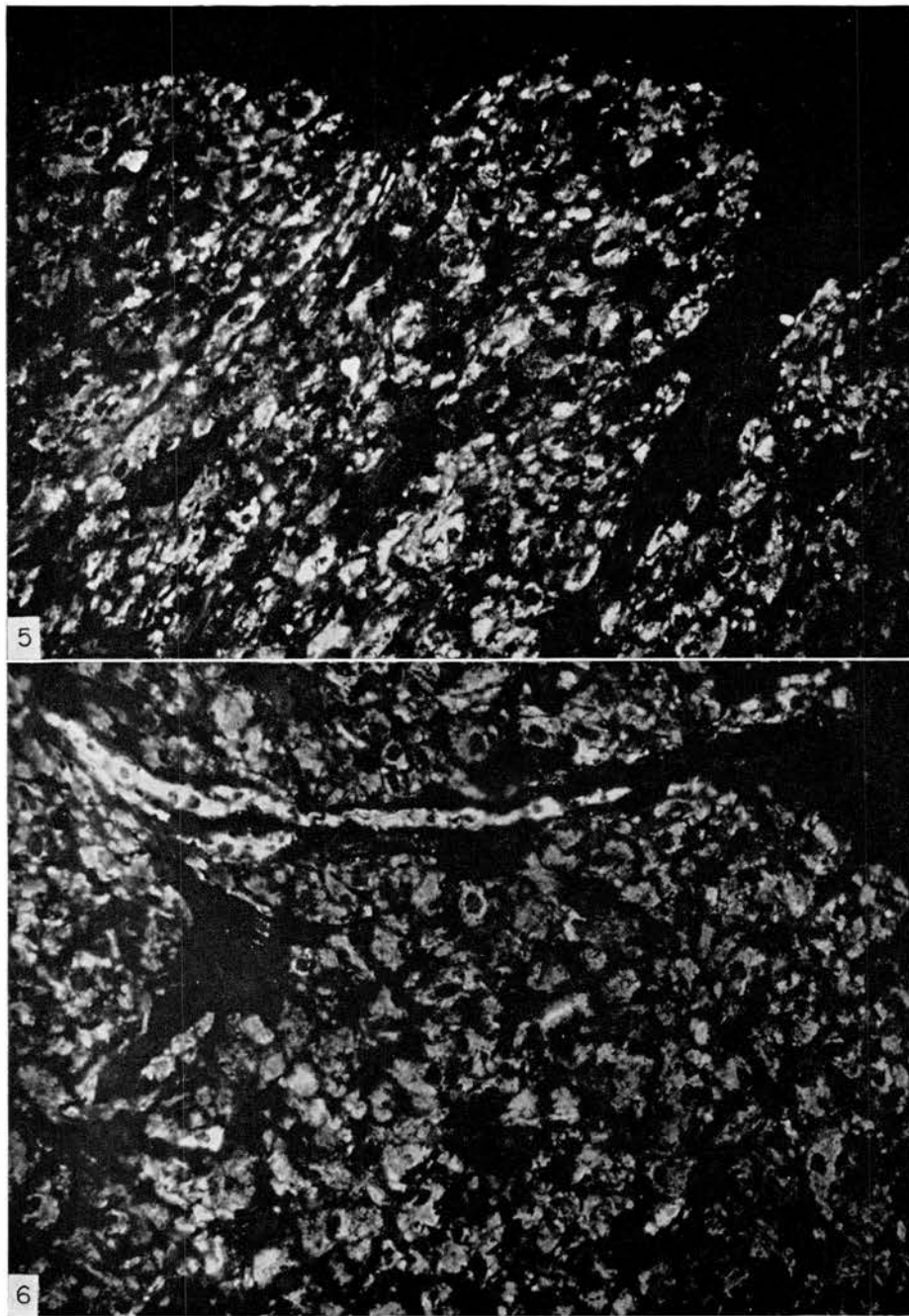


FIG. 5. Positive immunofluorescence staining of human corpus luteum given by serum A showing a clumpy pattern diffusely throughout the corpus luteum. Dark ground UV-blue light,  $\times 225$ .

FIG. 6. Same as Fig. 5, but showing in addition brilliant immunofluorescent staining of the theca cells in the trabeculae of the human corpus luteum. Dark ground UV-blue light,  $\times 250$ .

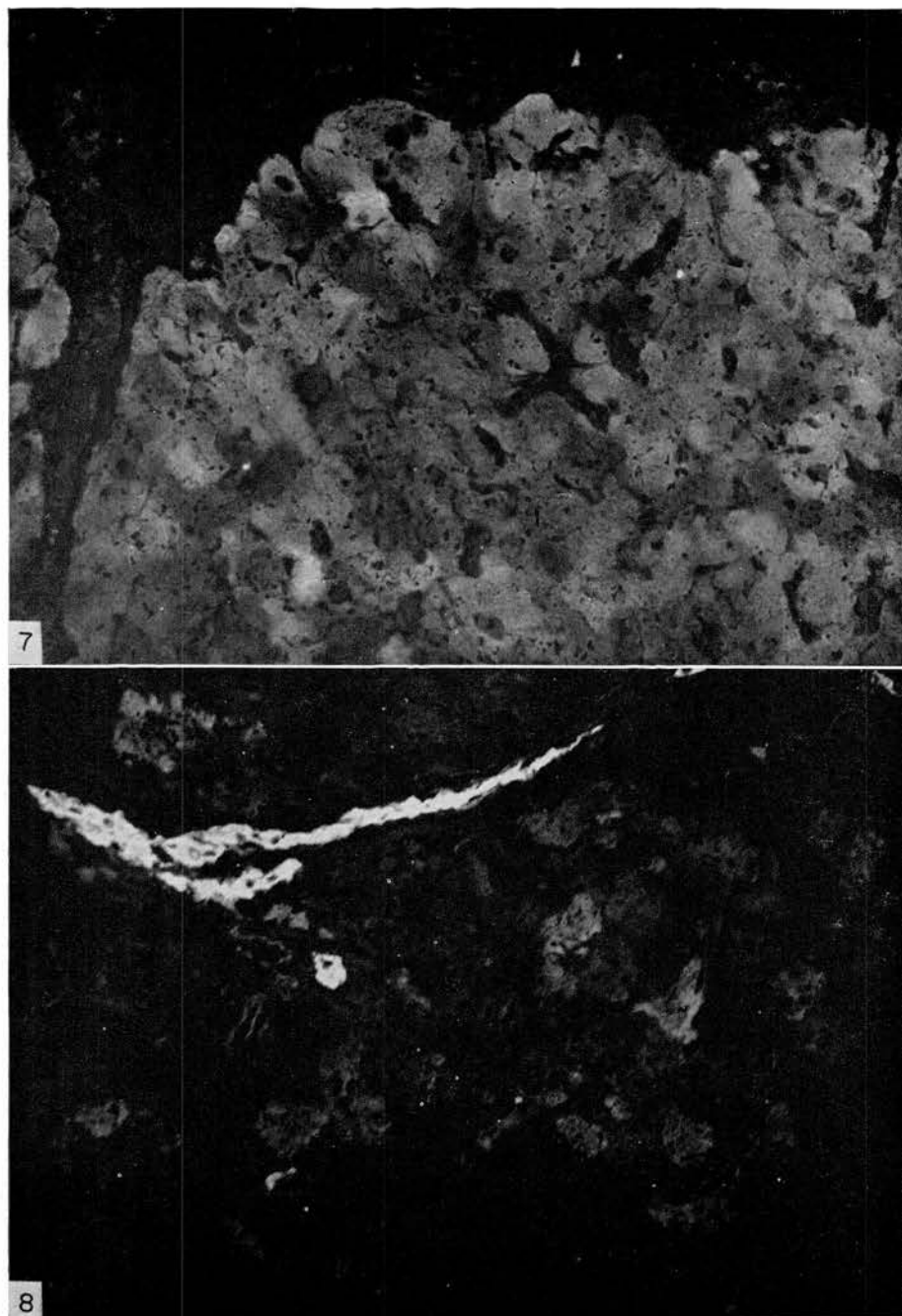


FIG. 7. Positive immunofluorescent staining of human corpus luteum given by serum F showing a confluent 'squamous-like' pattern throughout the corpus luteum. Dark ground UV-blue light,  $\times 240$ .

FIG. 8. Patchy immunofluorescent staining pattern of human corpus luteum and intense immunofluorescent staining of the theca cells in the trabeculae of the corpus luteum given by serum E. Dark ground UV-blue light,  $\times 240$ .

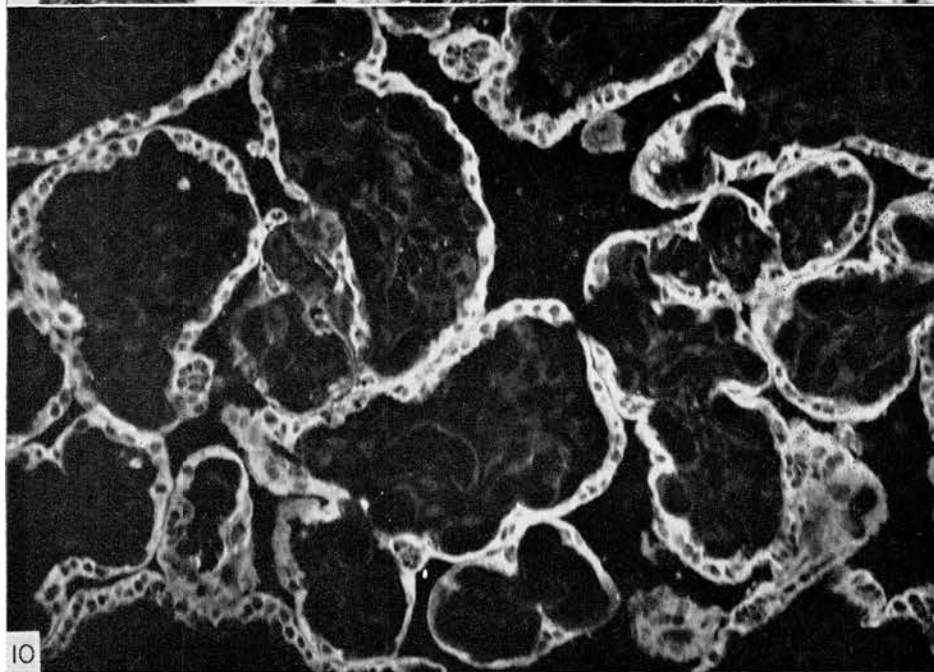
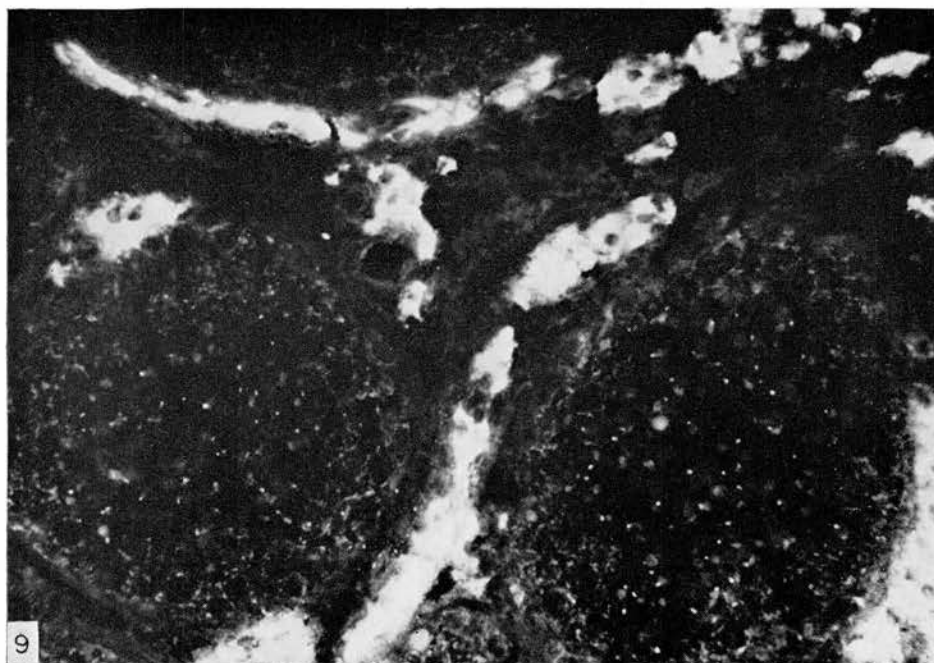


FIG. 9. Positive staining in the indirect immunofluorescent antibody technique of the interstitial cells and of the spermatids in rabbit testis given by serum A. Dark ground UV-blue light,  $\times 230$ .

FIG. 10. Positive staining of human placental trophoblasts in the indirect immunofluorescent antibody technique given by serum E. Dark ground UV-blue light,  $\times 240$ .



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Graafian follicles has been illustrated elsewhere (Irvine *et al.*, 1968). Some sera reacted with the interstitial cells of the rabbit ovary in the oestrus phase (Fig. 1). Marked hyperplasia of the interstitium is characteristic of the ovary of the pregnant or pseudopregnant rabbit. Sera A, G and J gave immunofluorescent staining of the hyperplastic interstitial cells while sera A, B, E, H and J reacted with a layer of granulosa cells in the same sections (Fig. 3). Interstitial cells were not conspicuous in the human ovarian tissue that was available. Fig. 4 shows that serum A, for example, also reacted with human ova. The tissue used in Fig. 4 was an ovarian biopsy from patient E (Irvine *et al.*, 1968; Irvine & Hartog, 1969).

Four clearly distinct immunofluorescent staining patterns were observed when human ovary in the luteal stage was used. These may be described as:

- (1) A clumpy staining pattern diffusely throughout the corpus luteum (Figs. 5 and 6).
- (2) A confluent squamous-like staining throughout the corpus luteum (Fig. 7).
- (3) A patchy staining of the corpus luteum involving scattered cells with varying intensity (Fig. 8).
- (4) An intense staining of a rim of cells at the periphery of the corpus luteum and in its trabeculae (Figs. 6 and 8).

The cells mentioned under reaction (4) did not form a continuous rim round the corpus luteum and the ovarian sections had to be carefully searched for their presence. It was thought that these cells represented the theca interna.

The results in Table I have been presented so as to group together the immunofluorescent staining patterns noted in relation to the human corpus luteum and its surrounding cells.

*Testis*

Seven of the ten sera showed a positive immunofluorescent reaction with the interstitial cells of testis. Six of these reacted with testis of human or rabbit origin, while one (serum G) reacted only with rabbit testis. An illustration of this type of immunofluorescent reaction is included in Irvine *et al.* (1968). A further example is shown in Fig. 9. Fig. 9 also shows positive immunofluorescence given by serum A with the spermatids of rabbit testis. Serum J also reacted with rabbit spermatids and with the interstitial cells of human or rabbit testis in the same manner as serum A.

*Placenta*

Fig. 10 illustrates a positive reaction with human placental trophoblasts. Such a reaction was observed with five of the ten sera.

*Adrenal*

All ten sera reacted positively by immunofluorescence with human adrenal cortex. Sera A, C and I showed only a weak but definite reaction, while sera B, D, E, F, G, H and J reacted strongly. Five of the ten sera stained all three layers of the cortex. Sera B, E, H and I stained only the lower part of the zona fasciculata and the whole of the zona reticularis (Fig. 11); they did not stain the zona glomerulosa or the upper part of the zona fasciculata. Serum G stained only the zona glomerulosa and fasciculata, but not the zona reticularis (Fig. 12). This difference in staining pattern was quite distinct and was confirmed by using contiguous sections of the same human adrenal gland for testing the different sera. Consistent immunofluorescent staining patterns were also obtained using sections from the adrenals of other patients.



The results of complement fixation tests with adrenal placenta and testis have been described previously (Irvine *et al.*, 1968).

#### *Absorption studies*

The results of absorption studies with buffered saline extracts of human adrenal and of human testis on four of the sera with steroid-cell antibodies reactive with extra-adrenal tissue (as well as with adrenal cortex) and on one serum (B.1493) from a patient with idiopathic

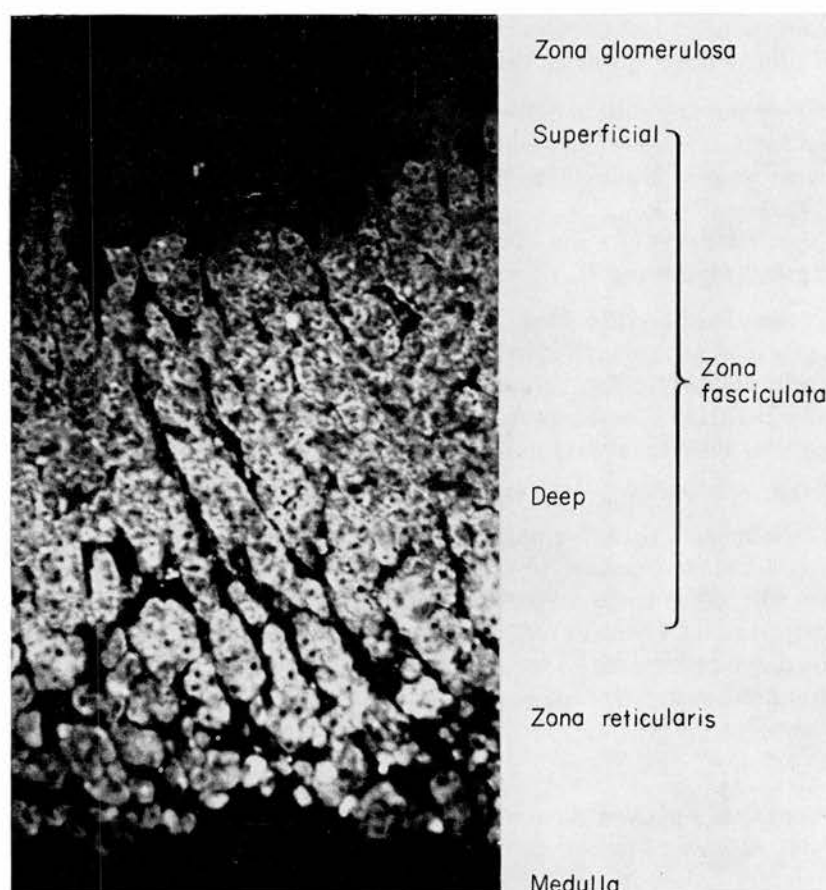


FIG. 11. Positive immunofluorescent staining of the human adrenal cortex confined to the lower part of the zona fasciculata and to the zona reticularis, but not involving the zona glomerulosa or the more superficial part of the zona fasciculata. Indirect immunofluorescent antibody method with serum E. Dark ground UV-blue light,  $\times 220$ .

adrenocortical insufficiency whose serum reacted only with adrenal cortex are shown in Table 2. With sera from patients A, B, D and E, adrenal extract absorbed out or significantly reduced the titre of antibodies reactive with human adrenal cortex, with the interstitial cells of human or rabbit testis, with the granulosa or theca cells of the follicular phase of the human ovary and of the rabbit ovary in oestrus, and with human placental trophoblasts.

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Human testis extract was similarly effective in absorbing antibodies reactive with these various tissues, indicating that there was a sharing of antigens between human adrenal, human testis and the other tissues studied. Extracts similarly prepared from human thyrotoxic thyroid tissue, from the mucosa of the body of human stomach and from rat liver had no significant effect on the immunofluorescent antibody titres shown by serum E with adrenal cortex, testis, ovary or placenta. Likewise, human testis extract had no effect on the

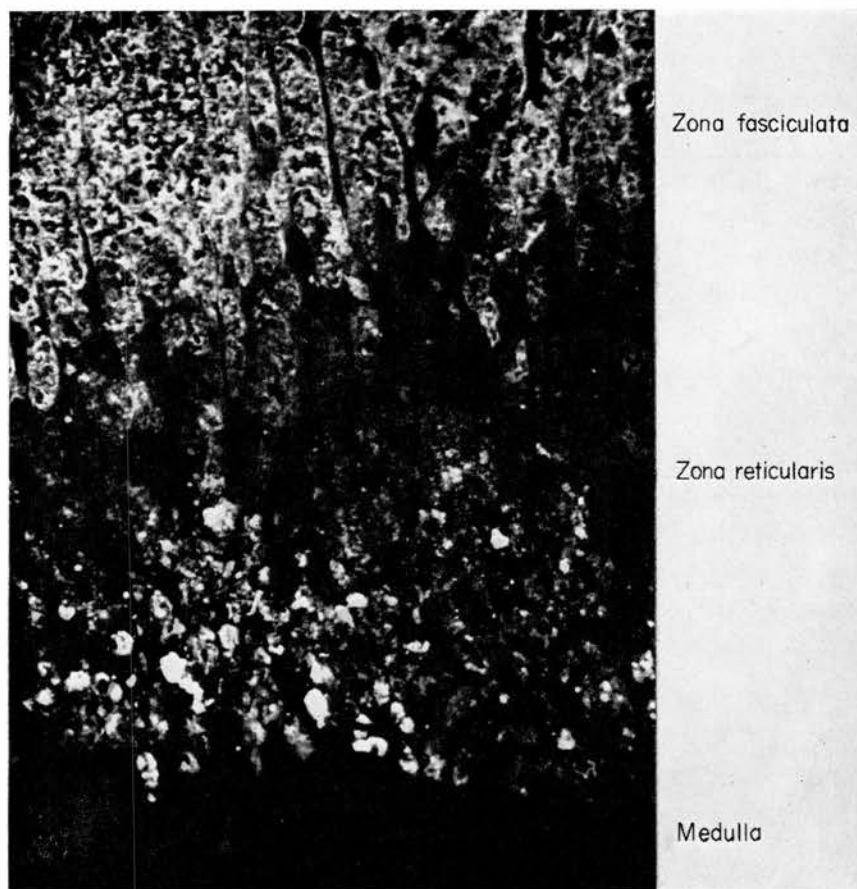


FIG. 12 Positive immunofluorescent staining of the zona fasciculata, but negative reaction with the zona reticularis of the human adrenal cortex given by serum G in the indirect immunofluorescent antibody technique. The clumpy staining of the zona reticularis is due to non-specific autofluorescence from lipid granules which fluoresce a reddish-brown as opposed to the specific green fluorescence in the zona fasciculata. Dark ground UV-blue light,  $\times 260$ .

immunofluorescent titre of serum B.1493 with adrenal cortex, indicating that the adrenal antibody or antibodies in this patient with idiopathic Addison's disease were distinct in their specificity from at least some of these antibodies reactive with adrenal cortex (and other steroid-producing tissues) in sera A, B, D and E.

The results of absorption studies on the various immunofluorescent reactions with human

TABLE 2. Results of absorption studies using extracts of human adrenal and of the human testis as well as control studies using extracts of thyroid, gastric mucosa and liver

Test tissue	Serum	Immunofluorescent titres					
		Before absorption	After absorption with extracts of:				
			Adrenal (human)	Testis (human)	Thyroid (human)	Gastric mucosa (human)	Liver (rat)
Adrenal cortex (human)	A	8	neg	neg			
	B	4	neg	neg			
	D	8	2	2			
	E	32	8	8	32	32	32
	B.1493	16	4	16			
Testis (human) interstitial cells	A	8	neg	neg			
	B	4	neg	neg			
	D	4	neg	neg			
	E	32	4	8	32	32	32
	B.1493	neg	neg	neg			
Testis (rabbit) interstitial cells	A	8	neg	2			
	B	4	neg	neg			
	D	4	2	neg			
	E	32	4	8	32	32	32
	B.1493	neg	neg	neg			
Ovary (human) follicular theca)	A	8	neg	2			
	B	8	neg	neg			
	D	8	2	2			
	E	128	16	32	128	128	128
	B.1493	neg	neg	neg			
Ovary (rabbit oestrus theca)	A	8	neg	2			
	B	2	neg	neg			
	D	2	neg	neg			
	E	32	8	16	32	32	32
	B.1493	neg	neg	neg			
Placenta (human)	A	4	neg	2			
	B	2	neg	neg			
	D	neg	neg	neg			
	E	neg	neg	neg	neg	neg	neg
	B.1493	neg	neg	neg			

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ovary in the luteal phase are summarized in Tables 3 and 4. While the diffuse clumpy staining of the corpus luteum given by sera A and C could be absorbed by either human adrenal or by human testis extracts, only adrenal was effective in absorbing the antibodies that gave the patchy staining (serum E) or confluent staining (serum F) of the corpus luteum (Table 3). Human adrenal extract absorbed the antibodies in sera A, C, D and E reactive with the theca cells at the periphery and in the trabeculae of the corpus luteum of

TABLE 3. Absorption studies on sera reactive by immunofluorescence with human corpus luteum

Patient	Pattern of immunofluorescence staining of corpus luteum	Reactivity absorbed by:	
		Human adrenal	Human testis
A	Clumpy: diffuse	Yes	Yes
C	Clumpy: diffuse	Yes	Yes
E	Patchy	Yes	No
F	Confluent	Yes	No

TABLE 4. Absorption studies on sera reactive by immunofluorescence with theca interna cells at the periphery of the corpus luteum in human ovary

Patient	Reactivity absorbed by:	
	Human adrenal	Human testis
A	Yes	Yes
C	Yes	Yes
D	Yes	No
E	Yes	No

human ovary but absorption with human testis extract was only effective in preventing this reaction given by sera A and C and was ineffective with regard to sera D and E (Table 4). These findings confirm that there must be a variety of steroid cell antigens only some of which are shared between the human testis and the ovary in the luteal phase, but that they may be all represented in one or more zones of the adrenal cortex. These findings also indicate that the theca cells surrounding the corpus luteum in human ovary contain more than one antigen.

## DISCUSSION

This paper establishes that there must be a complex of IgG antibodies reactive with adrenal cortex and with steroid-producing cells in the ovary, testis and placenta. An attempt has been made to characterize these different antibodies by their different immunofluorescent



staining properties. It has been demonstrated that antibodies reacting with the corpus luteum of human ovary cross-react with antigens in the adrenal cortex. It is evident that at least some of the cross-reacting antigens may not be evenly distributed between the three layers of the adrenal cortex. The antigens in the corpus luteum, responsible for the patchy immunofluorescent staining pattern given by sera B, E and H, are not present in the zona glomerulosa or upper part of the zona fasciculata while the antigens responsible for the confluent immunofluorescent staining pattern would appear not to be present in the zona reticularis. That the theca interna cells of the luteal phase of human ovary contain more than one antigen may be inferred from the observation that IgG antibodies in the sera of four patients reactive with this cell type could be absorbed by adrenal extract while an extract of human testis was only effective in absorbing this antibody or antibodies from the sera of two of these patients.

The analysis of the data in Table 1 is complicated by the fact that antibodies specific for components of the adrenal cortex, and not cross-reacting with other steroid-producing cells, are known to occur in some 50% of the sera of patients with idiopathic Addison's disease (Irvine *et al.*, 1968). Such antibodies have so far invariably reacted with all three layers of the adrenal cortex. These adrenal-specific antibodies are likely to be present in a proportion of the ten sera in the present study.

Although the number of sera available for study that reacted with steroid-producing cells in the gonads was small, there is a strong indication of three patterns of reactivity. Firstly, there is the pattern of immunofluorescent staining of all three layers of the adrenal cortex, diffuse clumpy staining of the human corpus luteum, positive reaction with theca interna cells surrounding the corpus luteum, reactivity with a layer of granulosa or theca cells in the rabbit Graafian follicles and a positive reaction with human placental trophoblasts. This contrasts with the second pattern which is characterized by patchy immunofluorescent staining of the human corpus luteum, positive reaction with theca cells surrounding the corpus luteum, positive reaction with granulosa or theca cells of the rabbit Graafian follicles and a reaction only with the deeper portion of the zona fasciculata and with the zona reticularis in the human adrenal cortex. The third pattern indicates that antigen at least in part responsible for the confluent staining pattern of human corpus luteum is not represented in the interstitial cells of the testis, or in the placental trophoblasts or in the zona reticularis of the human adrenal cortex.

From experience so far, it would appear that human corpus luteum is the most suitable tissue to use when screening sera for antibodies to steroid-producing cells in tissues other than adrenal. All ten sera in the present study were positive either in relation to the corpus luteum itself or the surrounding theca cells while only a proportion of the sera were positive with placenta, testis or ovary in the follicular phase. Human corpus luteum is simpler to obtain at termination of pregnancy than is human ovary in the follicular phase.

The remarkable correlation between the presence of gonadal antibodies and a clinical history of premature gonadal failure would suggest that immunological factors may have some pathogenic role in this condition. To date, with one exception (patient J), in female subjects it is only patients who have ovarian failure associated with adrenal autoimmunity that have been shown to have antibodies reactive with the steroid-producing cells of the gonads. The histological appearance of the ovaries on biopsy in patient E (Irvine *et al.*, 1968; Irvine & Hartog, 1969) was characterized by lymphocytic and plasma cell infiltration in the developing Graafian follicles. This would strongly suggest an autoimmune pathogenesis.

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By using frozen sections from this patient's ovarian biopsy, it was shown that her gonadal antibodies were truly autoimmune. As in other autoimmune diseases, the mediator of immunological damage may be lymphocytes, as suggested by the adoptive transfer experiments of McMaster & Lerner (1967) in relation to experimental chronic thyroiditis and of Levine & Wenk (1968) in relation to experimental chronic adrenalitis. Nerup, Andersen & Bendixen (1969) have demonstrated using the leucocyte migration test the existence of a state of organ-specific, anti-adrenal hypersensitivity of the cellular type in nine out of fifteen patients with idiopathic Addison's disease. Alternatively, there may be synergism between circulating antibody and lymphocyte-mediated antibody.

The present findings provide the first demonstration that an associated pathology (namely premature gonadal failure) in relation to what has generally been considered to be an organ-specific autoimmune disorder (namely idiopathic Addison's disease) may be due to cross-reacting antibodies.

## ACKNOWLEDGMENTS

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Antibody to the oxyphil cells of the human parathyroid in  
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## ANTIBODY TO THE OXYPHIL CELLS OF THE HUMAN PARATHYROID IN IDIOPATHIC HYPOPARATHYROIDISM

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### SUMMARY

IgG antibody to parathyroid oxyphil cells is described in the serum of one of nine patients with idiopathic hypoparathyroidism. This serum also reacted with the chief cells of the parathyroid. Whether other sera from the patients with hypoparathyroidism contained antibody to chief cells in low titre was uncertain.

### INTRODUCTION

Idiopathic hypoparathyroidism belongs to the 'club' of organ-specific autoimmune diseases along with the thyroid autoimmune diseases (thyrotoxicosis, Hashimoto goitre, primary atrophic hypothyroidism), idiopathic Addison's disease, Addisonian pernicious anaemia and certain cases of premature ovarian failure (Irvine, 1965; Blizzard, Chee & Davis, 1967; Irvine, Stewart & Scarth, 1967; Irvine *et al.*, 1968). Blizzard, Chee & Davis (1966) have described the presence of parathyroid antibodies as detected by the indirect immunofluorescent technique in 38% of seventy-four patients with idiopathic hypoparathyroidism, 26% of ninety-two patients with idiopathic Addison's disease, 12% of forty-nine patients with Hashimoto thyroiditis and in 6% of 245 control patients. No distinction was made between antibody to the chief (principal) cells or to the oxyphil cells of the parathyroid. The present paper describes antibody to the oxyphil cells and discusses some of the difficulties in the detection of antibody to the chief cells of the parathyroid.

### MATERIALS AND METHODS

A limited number of unfixed frozen sections were obtained from a small parathyroid adenoma from a female patient aged 42 years with hyperparathyroidism. Immediately after the removal of the adenoma from the patient, the tissue was rapidly frozen and the sections prepared. Parathyroid tissue was also obtained at routine post mortem from a male subject aged 50 years with no clinical evidence of parathyroid disorder. The sections were used in the

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indirect immunofluorescent antibody technique. The sera from nine patients with idiopathic hypoparathyroidism and from nine control subjects matched for age and sex were tested. Horse anti-human IgG conjugated with fluorescein isothiocyanate isomer I (British Drug Houses, England) with the fluorescein/protein ratio of 0.67 was used in a dilution of 1:6. The washing fluid in the immunofluorescent technique was veronal buffer, pH 7.2. Ten per cent glycerol in veronal buffer was used to mount a coverslip. Full details of the preparation of the conjugate and of the immunofluorescent staining are given elsewhere (Irvine, Chan & Williamson, 1969b). The sections were examined under a Leitz orthoplan microscope with a HBO 200 W lamp, primary filter BG 12 (5 mm), Tiyoda oil immersion supra wide angle dark ground condenser with numerical aperture 1.20 and secondary filter 530. The results were read subjectively.

Complement fixation tests were done using a phosphate buffer extract of a second and larger parathyroid adenoma (adenoma II) that had been stored at  $-20^{\circ}\text{C}$  for some months. None of the tissue from the second parathyroid adenoma had been snap frozen so that it was not suitable for immunofluorescence studies. The Takatsy microtitre technique (Irvine, 1966) was used with doubling dilutions of control and test sera and with dilutions of a veronal buffer extract of parathyroid adenoma II.

The parathyroid cryostat sections were also tested in the indirect immunofluorescent antibody technique with a serum (B.4320) known to contain a high titre of 'M' antibody (Doniach *et al.*, 1966). Control cryostat sections of rat kidney were included. Serum absorption experiments with a veronal buffer extract of rat liver were also carried out. One volume of rat liver was homogenized with 3 volumes veronal buffer (M.S.E. homogenizer). After centrifugation at 1750 g 10 min, the supernatant was used for absorption. One volume antigen and 1 volume serum were incubated for  $3\frac{1}{2}$  hr with continuous shaking at room temperature (Griffin Flask Shaker). After further centrifugation, the supernatant was used in complement fixation and immunofluorescence tests. Extracts of other tissues were used for absorption following the same procedure.

The hypoparathyroid and control sera were also tested against cryostat sections of human gastric mucosa, thyroid, adrenal, ovary and testis.

## RESULTS

The serum of one patient (B.8106) with idiopathic hypoparathyroidism showed clear immunofluorescent staining of the oxyphil cells of the parathyroid adenoma I (Fig. 1a) and of normal parathyroid (Fig. 1b). The patient was from Puerto Rico and her serum was made available to me through the kindness of Dr Lillian Haddock from the University School of Medicine, Puerto Rico, and Dr Robert Blizzard, The Johns Hopkins Hospital, Baltimore. She is patient J in the paper by Irvine, Chan & Scarth (1969a), and is also referred to in Irvine (1969a). She developed idiopathic hypoparathyroidism at 2 years and idiopathic Addison's disease at 19 years.

Serum B.8106 gave a negative reaction in the indirect immunofluorescent test for 'M' antibodies (Doniach *et al.*, 1966) when tested against rat kidney, but as described in Irvine *et al.* (1969a) gave a positive reaction in the indirect immunofluorescent antibody technique with all three layers of human adrenal cortex, certain steroid-producing cells in human or rabbit ovary, interstitial cells of human or rabbit testis, rabbit spermatids and with placental trophoblasts. Her serum was also positive for antibody to human gastric parietal cell in the

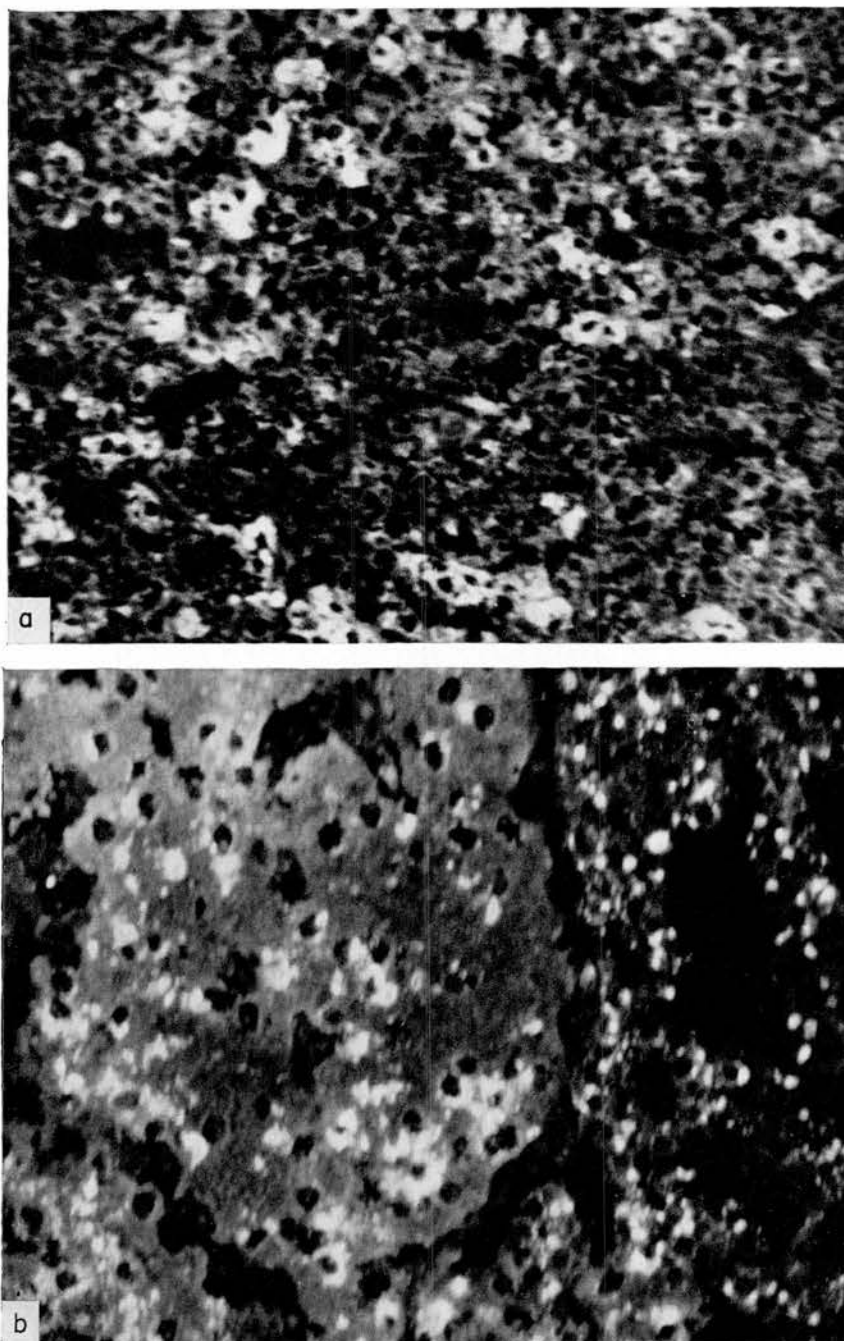


FIG. 1. (a) Positive reaction in the direct immunofluorescent antibody technique with the cytoplasm of scattered oxyphil cells in an unfixed cryostat section of a parathyroid adenoma using serum B.8106 from a patient with idiopathic hypoparathyroidism and anti-human IgG-FITC conjugate. The cytoplasm of the parathyroid chief cells is also considered to show positive immunofluorescent staining. Dark ground UV-blue light,  $\times 185$ . (b) Same as (a) but with normal post-mortem parathyroid. In this instance the oxyphil cells are present in a discrete cluster. The bright globules are due to redish orange autofluorescence and not to the green fluorescence of the specific staining of the cytoplasm of the parathyroid cells. Dark ground Ultraviolet-blue light,  $\times 400$ .

indirect immunofluorescent technique, but gave a negative immunofluorescence test for antibody to human thyroid epithelial cytoplasm.

Complement fixation tests with serum B.8106 were negative with rat liver and with human thyroid extracts, but positive with an extract of mucosa from the body of human stomach. The complement fixation tests with parathyroid adenoma II were equivocally positive on chessboard titration at a serum dilution of 1:4 and an antigen dilution of 1:4. Serum B.8106 was the only one to give even this degree of reaction in the chessboard titration with a buffered saline extract of parathyroid adenoma II out of the eighteen sera so tested.

Serum B.4320, which was known to contain high titres of 'M' antibody, was tested against cryostat sections of parathyroid adenoma I and of the normal parathyroid. Positive immunofluorescent staining of the cells with comparable distribution to the oxyphil cells stained by serum B.8106 was obtained and the fluorescence was of similar intensity. However, serum B.4320 gave an intense staining of the renal tubules and of the liver parenchyma when tested against rat kidney and liver sections, while serum B.8106 gave a negative reaction with these tissues.

The immunofluorescent staining of the oxyphil cells in the sections of normal parathyroid could not be absorbed out by rat liver extract. The titre of the IgG antibody to oxyphil cells was 1:16 before and after absorption. In contrast, the titre of serum B.4320 with the oxyphil cells in the normal parathyroid sections was reduced from 1:256 to 1:64 by prior absorption of this serum with liver extract.

Serum B.8106 also gave a distinct and consistent increase in the brightness of fluorescence of the chief cells in cryostat sections of parathyroid adenoma I and of the normal parathyroid. The titres of the reaction with the parathyroid chief cells given by serum B.8106 and by serum B.4320 before and after absorption with liver extract were comparable to those recorded, respectively, for the reaction with the parathyroid oxyphil cells. Three of the other eight sera from patients with idiopathic hypoparathyroidism and one of the nine control sera were also considered to give a positive indirect immunofluorescence test with the chief cells of parathyroid adenoma I but the results were not consistent when checked with sections of the normal parathyroid. The interpretation of a positive or negative immunofluorescence reaction with the chief cells presented some difficulty. This was because there was no clear contrast within the specimen itself as in the case of the oxyphil cells and it was necessary to assess whether the general degree of fluorescence in the cytoplasm of these cells in each section was greater or equal to that of the controls, there being some slight degree of background staining.

The control serum (B.4752) that appeared to give a positive indirect immunofluorescence test for the cytoplasm of the chief cells in parathyroid adenoma I was shown to contain antibody to human parietal cells and to human thyroid epithelial cytoplasm but was negative for 'M' antibodies and for antibodies to steroid-producing cells in the gonads and placenta. One other control serum gave a positive immunofluorescence test for gastric parietal cell antibody, but otherwise the control sera were all negative.

## DISCUSSION

The occurrence of antibodies that are specific for one or each of the cell types in the parathyroid is what one would anticipate in the sera of at least a proportion of patients with idiopathic hypoparathyroidism. As already mentioned, this condition is associated clinically



with other disorders that are characterized by the formation of autoantibodies that are highly specific for components of individual tissues. The histology of idiopathic hypoparathyroidism is that of atrophy of the parathyroid cells together with lymphocytic infiltration (see Irvine, 1969b). Lupulescu *et al.* (1968) have demonstrated that repeated inoculation of homologous parathyroid tissue into dogs may induce isoimmune hypoparathyroidism with the characteristic biochemical and histopathological features and the presence of complement fixing parathyroid antibodies in low titres in the serum. The isoimmune hypoparathyroidism in dogs was similar to that described by Lupulescu *et al.* (1965) in rats.

Serum B.8106 which gave a positive immunofluorescence test with the oxyphil cells in parathyroid adenoma I and in the normal parathyroid also reacted positively with human gastric parietal cell cytoplasm and with steroid-producing cells in the gonads. The negative immunofluorescence reaction with rat kidney tubules and with rat liver sections and with human thyroid epithelial cytoplasm and the negative complement fixation tests with rat liver indicate that the positive immunofluorescence reaction with the oxyphil cells was not due to the presence of tissue non-specific 'M' antibodies. This is confirmed by the failure of liver extract to absorb the antibody reacting with parathyroid oxyphil cells. The distinction from 'M' antibodies is an important point to establish as the oxyphil cells are known to be rich in mitochondria and it has been demonstrated in this paper that 'M' antibodies can give positive immunofluorescence tests with parathyroid sections in the distribution of the oxyphil cells. Also in favour of the immunofluorescence reaction of serum B.8106 being specific for parathyroid is the observation that 'M' antibodies are rare in the organ-specific group of diseases (Doniach *et al.*, 1966).

The application of microphotometry (Irvine *et al.*, 1969b) may allow a more confident distinction to be made between positive and negative immunofluorescent reactions with the chief cells of parathyroid tissue. The findings in the limited series reported here are in keeping with those reported in a much larger series by Blizzard *et al.* (1966). However, in Blizzard's series it is anomalous, in view of the rarity of idiopathic hypoparathyroidism, that as many as 6% of this control subjects were positive for parathyroid antibodies presumably to chief cells. It is also anomalous that the suitability of parathyroid adenomata for fluorescence studies could not be predicted and that no confirmatory reports have so far appeared. Clearly the incidence and characterization of parathyroid antibodies requires further study. It is not possible on the present evidence to state whether the parathyroid oxyphil and chief cells have distinct autoantigens one from the other.

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JUVENILE FAMILIAL ENDOCRINOPATHY

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### SUMMARY

A case of primary hypothyroidism, idiopathic Addison's disease, idiopathic hypoparathyroidism (with preceding moniliasis), Addisonian pernicious anaemia and primary ovarian failure is described. She died at the age of 24 years following an illness compatible with adrenal crisis. At post-mortem there was no recognisable adrenal or ovarian tissue; there was only a minute portion of probable parathyroid tissue and the uterus was infantile. Her serum contained antibodies reactive with adrenal cortex, steroid-producing cells in the gonads, placental trophoblasts and thyroid epithelial cytoplasm and intrinsic factor.

Her brother, who was known to have gluten enteropathy, died aged 11 years following an illness compatible with adrenal crisis. His adrenal glands were grossly atrophic at autopsy.

The parents were consanguineous and both showed either clinical or serological evidence of organ specific autoimmune disease.

### INTRODUCTION

The following two cases and family study are described in view of the rarity of adrenal insufficiency in siblings and to record the details of a case of primary ovarian failure associated with ovarian autoimmunity with streak ovaries and with polyendocrinopathy.

### CASE 1

#### History

M. L. Female, Born 12.1.1945. Her birthweight was  $8\frac{1}{2}$  lbs. (3.7 kilos), the outcome of a normal pregnancy and delivery. Her growth and development progressed normally to the age of 5 years, when she first developed patchy alopecia with depilation of the eyebrows and eyelashes. At 7 years, she spent ten months in a fever hospital with suspected tuberculous



meningitis, which was never proven. On discharge from hospital, she was underweight and the alopecia had become complete. Except for one year, at about the age of 9 years, when there was some regrowth of hair, the alopecia remained unchanged. Finger-nail dystrophy was first noted during her stay in the fever hospital and persisted thereafter.

In 1960, at the age of 15 years, she was referred to the Endocrine Clinic of the Mater Misericordiae Hospital, Dublin, by Dr J. Barnes of the Dermatology Department. She was of small stature ( $49\frac{1}{4}$  inches (124 cms) in height; 61 lbs. (27 kilos) in weight). Her skin was dry and cold. She had a red, raw tongue, showing patches of moniliasis. There was marked moniliasis of the finger nails and alopecia totalis.

There was no history of tetany or steatorrhoea. The menarche had not occurred. The external genitalia were infantile and there was no breast development. The systolic B. P. was 92 mms. Hg.

The Haemoglobin was 62% (9gms%), the total white cell count and differential count and urinalysis were normal. There was no excess of fat in the stools. Her bone age was only 8 years in contrast to her chronological age of 15 years, but there was no evidence of osteoporosis. The serum cholesterol was 288 mgs. % and an E.C.G. showed low voltage and prolongation of the Q. T. interval consistent with hypothyroidism. She was considered to have primary hypothyroidism and treatment was begun with thyroid extract 2 grs. (130 mgms) per day. This was later replaced by l-thyroxine 0.2 mgms. per day.

In September 1970, the 24 hour urinary 17-ketosteroid and 17-ketogenic steroid excretion was 1.5 mgms. and 0.8 mgms., respectively. At about this time she had an episode of collapse with B. P. = 60/45 mm. Hg, blood sugar = 44 mgms% and serum sodium = 120 mEq/l. She responded to intravenous dextrose and the blood pressure rose to 100/65 mm. Hg. A diagnosis of primary idiopathic adrenal insufficiency was made and her general health was much improved by the addition of steroid replacement therapy.



In December 1961, she had her first episode of tetany (serum calcium = 5.4 mgms%). The response to i. v. calcium gluconate was rapid. Steatorrhoea was again excluded by stool analysis, d-xylose test and jejunal biopsy. She was considered to have primary hypoparathyroidism. Calcium lactate and vitamin D were added to her therapy.

In October 1962 (at the age of 17 years) her bone age was 13 years, height 56 inches (142 cms) and weight 72 lbs. (32 kilos). Serum calcium = 10.7 mgms%, phosphorus = 4.3 mgms%, alkaline phosphatase = 11 K.A. units, haemoglobin = 84% (12.2 gms%). The total 24 hour urinary gonadotrophins estimated by the mouse uterus test was between 26 and 52 mouse units. The subsequent progress was complicated by a rise in the serum calcium to 12.1 mgms% and the development of a ureteric calculus which was passed spontaneously. The dosage of calcium and vitamin D were adjusted.

In September 1966 a macrocytic anaemia and histamine-fast achlorhydria were demonstrated. The serum vitamin B<sub>12</sub> was 100 pg./ml. and the Schilling test was 0.05% rising to 23% when repeated with oral intrinsic factor.

In 1968, at the age of 23 years (height 61 inches (154 cms), weight 81 lbs. (36 kilos), bone age = 15 years) she had never menstruated and there was no palpable breast tissue. Gynaecological examination was refused. She was Barr positive on buccal smear and chromosome studies by lymphocyte culture gave normal results, 46 XX (Dr Patricia Jacobs, M.R.C. Clinical and Population Cytogenic Unit, Edinburgh).

Her clinical history is summarised in Table 1. This patient is patient B in the paper by Irvine *et al.*, (1968) and patient No. 3 of Table IV and No. 2 of Table V in Irvine (1970a).

#### Serological findings

The results of her antibody studies on serum obtained in 1966 and subsequently are shown in Table 2. The details of the serological techniques

TABLE 1SUMMARY OF THE MAIN CLINICAL FEATURES OF PATIENT M.L.

Normal delivery and birthweight	
Alopecia at	7 years
Moniliasis at	7 years
Primary hypothyroidism at	15 years
Primary adrenocortical failure (probably idiopathic) at	15 years
Primary hypoparathyroidism at	16 years
Addisonian pernicious anaemia at	22 years
Complete failure of menstruation with no menarche and no secondary sex characteristics at	23 years

TABLE 2  
SEROLOGICAL RESULTS FOR PATIENT M.L.

<u>TISSUE</u>	<u>Staining pattern*</u>	<u>Immunofluorescence</u>	<u>METHOD</u>	<u>Other</u>
<u>Ovary</u>	Human corpus luteum	Clumpy Confluent Patchy Theca cells	neg neg + ++	
	Follicular phase	Granulosa cells/theca Ova	++ +	
<u>Rabbit Ovary</u>		Granulosa cells/theca Hyperplastic interstitial cells Ova	++  neg neg	
<u>Testis</u>				
	Human	Interstitial cells Spermatids	+ neg	
	Rabbit	Interstitial cells Spermatids	++ neg	
<u>Placenta</u>				
	Human	Trophoblasts	+	



(TABLE 2 CONTINUED)

TISSUE	METHOD		
	Staining pattern*	Immunofluorescence	Other

<u>Adrenal</u>	Human	Glomerulosa	neg )	G. F. T. = 8
		Fasciculata	++ )	
		Reticularis	++ )	

Parathyroid

Human adenoma

neg

Thyroid

Human

Cytoplasm  
Thyroglobulin

++

G. F. T. = 16  
T. C. H. = 250Stomach

Body of human stomach

Parietal cells

neg

G. F. T. = neg

Human gastric juice

Radioimmunoassay for  
intrinsic factor anti-  
body I = 880 mg/units/mlMiscellaneous

Rat liver

Mitochondria

neg

Rat kidney

Nuclei

neg

Tissues listed above

C. F. T. = complement fixation test.    T. C. H. = tanned cell haemagglutinin test  
for antibody to thyroglobulin.

\* as described in Irvine et al., 1969.



employed were as previously described (Irvine et al., 1967; Irvine, Chan and Scarth, 1969) using undiluted serum and anti-human IgG-F.I.T.C. conjugate in the immunofluorescence technique and 2 MHD of complement and 50% haemolysis as end-point in the complement fixation test.

### Autopsy

While on holiday in the West of Ireland in August 1969 the patient complained of pyrexia, vomiting and diarrhoea with cramp-like abdominal pain. These symptoms worsened over the following 12 hours and she lapsed into unconsciousness and died in the ambulance on the way to the Mater Misericordiae Hospital.

#### Macroscopic findings:

The body was completely devoid of hair and the finger-nails were thickened, longitudinally ridged and reduced to approximately one-third in area. Some pigmentation was noted at the margins of the tongue and on the gums. The thyroid weighed 4 grms and the thymus 7 grms. The adrenals could not be detected macroscopically, the spleen was absent and rudimentary ovaries and uterus were identified. Other structures were grossly normal.

#### Histology:

Pituitary gland: normal, with abundant cells in the anterior pituitary of normal appearance and variety, using H and E stains.

Thyroid: the glandular tissue was almost totally replaced by acellular fibrotic tissue. Numerous small foci of surviving thyroid glandular tissue were evident throughout the fibrotic mass. These foci were surrounded and infiltrated by an intense localised lymphocytic infiltrate similar to that seen in Hashimoto thyroiditis.

Parathyroids: only one small focus (2 mm) of probable parathyroid tissue was identified after a careful search.

Adrenal glands: no recognisable adrenal tissue was detectable in multiple sections from the fatty tissue of both adrenal areas. A focus of old haemorrhage in the form of haemosiderin deposit was found in the right adrenal area.

Ovaries: represented by thin plaques of fibrotic tissue in which follicles were not found.

Uterus: the cavity was lined by columnar epithelium resembling the normal endo-cervical type mucosa. No formed endometrium was detected.

Thymus: normal with reasonable cortico-medullary differentiation and no conspicuous germinal centre formation in the medulla.

Pancreas: normal with normal islet tissue.

Kidneys: normal apart from some small foci of calcification present in the epithelium of occasional tubules.

## CASE 2

### History

S. L. Male, Born 1947. Brother of Case 1. He was investigated at 7 years of age for coeliac disease and moniliasis of the finger-nails and mouth. His height then was 47 inches (118 cms) and weight 34 lbs. (15 kilos). Steatorrhoea was confirmed and a gluten-free diet was started with symptomatic improvement.

At the age of 11 years, patchy alopecia of the scalp was noted. Later the same year, he was admitted to hospital with vomiting and diarrhoea of two days duration. He was underweight with monilial infection of the popliteal fossa of the right leg, of both hands including the nails, and of the mouth. The tongue was bald and red. The serum calcium was 8.5 mgm% and the phosphorus was 4.5 mgms%. The faeces again contained a large excess of free fatty acid and occasional globules of neutral fat. His condition improved on a strict gluten-free diet and the serum calcium levels rose to 10.4 mgms% and 10.8 mgms%. Six months later he was re-admitted to hospital with a history of intermittent abdominal pain and vomiting of 10 days duration. There was no diarrhoea on this occasion. He collapsed and died within an hour of admission to hospital.

### Autopsy

At post-mortem, moniliasis as described was noted. Both adrenal glands were extremely thin and atrophic. The thyroid and pituitary glands were macroscopically normal and the parathyroids could not be identified. There was marked enlargement of the mesenteric lymph nodes. Histology of these tissue was not available.

### FAMILY HISTORY

The parents of M. L. and S. L. were first cousins. They had two other children, one of whom was reputed to be in excellent health. The other died in childhood of gastroenteritis during an epidemic in 1946.

The sera of both parents contained gastric parietal-cell antibodies by the indirect immunofluorescence test, and the serum of the mother (but not the father) was weakly positive for intrinsic factor antibody I (5.5 mg. units/ml.). Both sera were negative for thyroid cytoplasmic antibodies and for thyroglobulin antibodies and were also negative for antibodies reactive with adrenal cortex, ovary (graafian follicles and corpus luteum), testis, placenta, mitochondria and nuclei.

The surviving sibling had emigrated and was not available for clinical or serological assessment.

In March 1968 the father (aged 70 years) was found to have a macrocytic anaemia and achlorhydria and an abnormal Schilling test indicative of vitamin B<sub>12</sub> malabsorption. His general condition improved rapidly with parenteral vitamin B<sub>12</sub> therapy.

The father's sister attends the Mater Misericordiae Hospital with diabetes mellitus and Addisonian pernicious anaemia.

The available data concerning this family are summarised in Fig. 1.



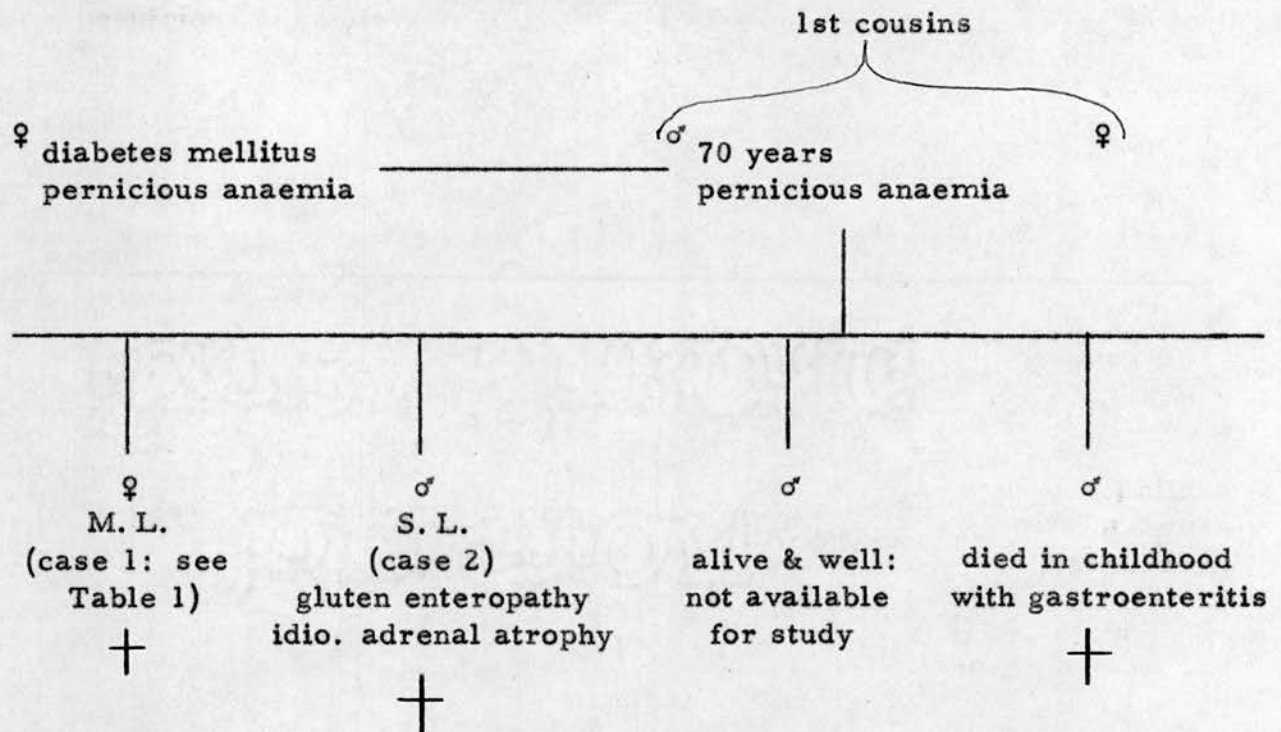


Fig. 1. Summary of family history.



## DISCUSSION

In spite of the absence of more precise modern methods of investigation of adrenal function, there can be little doubt of the diagnosis of idiopathic adrenal insufficiency in the case of M. L. and of S. L. In M. L. primary adrenal atrophy and primary thyroid atrophy (with lymphocytic infiltration) were shown unequivocally at autopsy. Such severe atrophy would not be produced simply by replacement doses of oral steroids or of thyroxine. The fact that only remnants of parathyroid tissue could be found after a careful search at autopsy (both macroscopically and microscopically) confirmed the clinical diagnosis of idiopathic hypoparathyroidism. In her brother S. L. the severe atrophy of the adrenals noted at autopsy was in keeping with the clinical diagnosis of primary adrenal insufficiency.

The association of alopecia, moniliasis, primary hypothyroidism, primary hypoparathyroidism and Addisonian pernicious anaemia with idiopathic adrenal failure has been documented and reviewed (Carpenter et al., 1964; Blizzard, Chee and Davis, 1967; Irvine, Stewart and Scarth, 1967; Irvine, 1968 and 1970 a & b). The association of malabsorptive disease with idiopathic Addison's disease has been described by Craig, Schiff and Boone (1955), Whitaker et al. (1956), Morse et al., (1961) and Goudie et al., (1969). The association of ovarian failure with idiopathic Addison's disease has been described clinically by Turkington and Lebovitz (1967) and both clinically and immunologically by Irvine et al., (1968, 1969) and by Irvine (1970 a & b). A phenomenon that is shared by many of these conditions is the occurrence of auto-antibodies in the sera that are specific for constituents of the organ that is the site of the disease (e.g. thyroid, parathyroid, gastric mucosa and adrenal cortex). The situation regarding the ovary is different in that there is a sharing of antigen between the steroid-producing cells of the ovary and the steroid-producing cells of the adrenal cortex (Irvine et al., 1969). Patients who develop an immune response to steroid cell antigens that are present in the adrenals and also in the gonads are liable to have

combined adrenal and ovarian failure, whereas patients who develop an immune response to steroid cell antigens that are only present in the adrenals do not develop ovarian failure in addition to adrenal atrophy. Extra-adrenal steroid cell antibodies are generally absent from the sera of patients with ovarian failure alone and without idiopathic Addison's disease (Irvine, 1970b). An attractive hypothesis is that the association of clinical diseases described above (and exemplified particularly in Case M. L. of the present paper) is accounted for by a genetically determined disorder of immunological tolerance (Irvine, 1964; Irvine et al., 1967).

Patient M. L. of the present paper with idiopathic Addison's disease and antibodies in the serum reactive with ovarian steroid-producing cells is now the second case in the literature in whom ovarian failure has been shown unequivocally to be associated with streak ovaries. Both patients were normal Barr positive on buccal smear and both had normal chromosome patterns on lymphocyte culture. The other case was described by Irvine et al., (1968) and by Kolb et al., (1970). While the possibility of mosaicism cannot be entirely excluded, it would seem likely that the destruction or failure of development of the ovary in these cases may be due to the development of autoimmunity (humoral and cellular) to ovary at an early age, i. e. before puberty. In patient (M. L.), and also in Dr Felix Kolb's patient, there was abundant clinical evidence that frank autoimmune disease had developed before puberty and it is therefore probable that the ovary may have been implicated at that time. These two cases, together with the finding that the sera of 49 patients with gonadal dysgenesis and chromosomal defects but without associated Addison's disease were all negative for steroid cell antibodies (Price and Irvine, 1969), should suffice to question whether streak ovaries are always associated with chromosome abnormalities. In some instances streak ovaries may perhaps result from autoimmune mechanisms at an early age.

In patients with idiopathic Addison's disease and ovarian failure (with adrenal and extra-adrenal steroid cell antibodies) but in whom a menarche

has occurred, the ovarian histology is characterised by lymphocytic infiltration in relation to the developing ovarian follicles (Irvine et al., 1968; Bayliss and Irvine, 1970).

The combination of idiopathic Addison's disease with hypothyroidism (Schmidt's syndrome) is sometimes familial and is thought to be an autosomal recessive character (Carpenter et al., 1964; Beaven et al., 1959). Dunlop (1963) has stated that all cases of familial Addison's disease have so far proved to be idiopathic if autopsy was performed. Idiopathic Addison's disease has been described in two pairs of identical twins (Smith et al., 1963; Heggarty, 1968). Familial aspects of Addison's disease have also been reported by Hung, Migeon and Parrot (1963) and by Meechen and Wynn (1967). Spinner, Blizzard and Childs (1968) made an analysis of the history from 140 families containing patients with idiopathic Addison's disease without idiopathic hypoparathyroidism, Addison's disease with hypoparathyroidism and hypoparathyroidism alone. They demonstrated a significantly greater similarity of clinical and other attributes among affected persons within the families than among unrelated persons. Some cases were apparently genetically determined and the distribution of affected persons within the family was compatible with an autosomal recessive pattern. Other cases did not fit any Mendelian pattern even although their families contained more than one affected person.

The present family would be compatible with a genetic tendency to autoimmunity with both parents carrying the trait as evidenced by clinical pernicious anaemia in the father and his sister, and the presence of parietal-cell antibodies in the serum of both the father and mother. The fact that both parents carried the trait may explain why the manifestations of autoimmunity were so pronounced and occurred so early in their children. The fact that the parents were consanguineous would also greatly strengthen the transmission of a recessive trait or a dominant trait with incomplete penetrance.



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PRIMARY HYPOPARATHYROIDISM, ADDISON'S DISEASE  
AND OVARIAN FAILURE

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"Reproductive Endocrinology" (1969) Symposium of Endocrine  
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## PRIMARY HYPOPARATHYROIDISM, ADDISON'S DISEASE AND OVARIAN FAILURE

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M.H.,\* a baby girl born at full term in 1948, of normal weight and of normal pregnancy and spontaneous delivery, was in good health until two years of age when she developed stunting of growth, a protuberant abdomen and passage of large foul stools. A tentative diagnosis of sprue syndrome was made. There appeared an urticarial rash and frequent respiratory infections, and, finally convulsive seizures improved by calcium administration. A diagnosis of idiopathic hypoparathyroidism was established with findings of a serum calcium of 5.9 mg. per cent and serum phosphate of 7.6 mg. per cent. Injection of parathyroid hormone produced phosphaturia (Ellsworth-Howard test positive). Treatment with oral calcium, a low phosphate diet and dihydrotachysterol (A.T.10) controlled her seizures for about three years. In 1954, at six years of age, seizures returned requiring injections of parathyroid extract with good response. Subsequently two foetal parathyroid transplants were attempted with apparent improvement, although her previous medications had not been stopped until 1956 when she developed mild hypercalcaemia. Her physicians reported the transplants to be successful (Akers *et al.*, 1958). Shortly thereafter, however, hypocalcaemic tetany recurred.

She was first admitted to the University of California Medical Center in 1958, at the age of 10, following an acute tetanic seizure with choking of the optic discs, severe hypocalcaemia and hyperphosphatemia. Marked skeletal demineralization, a retarded bone age and malformation of her teeth and hips were noted. The diagnosis of hypoparathyroidism was confirmed but the nature of her bone disease

\* This patient is No. 2 of Table III and No. 3 of Tables IV and V of the preceding paper.

remained obscure. Following treatment with vitamin D and aluminium hydroxide gel, a third transplant of adenomatous parathyroid tissue placed into four millipore chambers was attempted. Since serum calcium rose to levels of mild hypercalcaemia, vitamin D was discontinued four weeks later, while oral calcium and the low phosphate diet were continued.

Six months after the transplant hypocalcaemia returned when oral calcium was stopped and a high phosphate diet was given. Vitamin D therapy was resumed and the patient felt well until the age of 12 years, 6 months when she was readmitted with nausea, weakness and deep pigmentation of her skin and mucous membranes. A small goitre was noted as well. Hypercalcaemia was present and a diagnosis of primary Addison's disease was established because of urinary hydroxycorticoids of 1.6 mg./24 hours, absent ketosteroids and failure to rise after intravenous infusion of 25 units of ACTH over eight hours. The protein bound iodine level was 9.8 micrograms per cent, radioactive  $T_3$  uptake of the red cells 18 per cent (Normal 13 to 20) and radioiodine uptake over the thyroid gland 4 per cent in 24 hours with rise to 25 per cent after 5 units of thyrotropic hormone for two days. A diagnosis of probable thyroiditis was made rather than primary myxoedema, although thyroglobulin antibody levels were negative. The urinary gonadotrophin levels were elevated and an X-ray survey by Dr H. Steinbach suggested the diagnosis of gonadal dysgenesis. Following hydration and hydrocortisone administration, serum calcium promptly returned to normal and the patient rapidly returned to a state of well-being. The four millipore chambers were removed showing scar tissue and absence of viable parathyroid cells.

The patient was well for the next two years taking vitamin D and hydrocortisone; the small goitre disappeared spontaneously. Her height at 10 years was 127 cm. and at 12 years was 137 cm. *i.e.* below the third percentile. In 1964 she was readmitted to hospital because of failure to mature and the finding of a markedly retarded bone age. Buccal smears were chromatin positive. Urinary gonadotrophin levels were positive at 5 and negative at 80 mouse units. Insulin tolerance test showed normal sensitivity. Gastric analysis failed to show free acid before and after histamine stimulation. The Schilling test was normal. Stool fat excretion was normal. Her serum was weakly positive for thyroid microsomal antibody by immuno-

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fluorescence and was definitely positive for antibody to adrenocortical cells and to parietal cells by immunofluorescence. Her serum was also positive for intrinsic factor antibody I, using albumin-coated charcoal radioimmunoassay. These findings suggested that her endocrine deficiency state may be due to autoimmune disease (Kolb *et al.*, 1965). Thyroxin was given at first to promote growth. Vaginoscopy showed a small cervix and rectal examination a cord-like uterine body. Chromosomal analysis (Dr D. Lindner) showed 46 chromosomes with XX pattern. Small doses of oestrogen were given. In 1966, after a brief hypocalcaemic seizure, the patient fractured her left hip, but X-ray examination showed a previous spontaneous fracture of the right hip as well. After immobilization in a hip spica a wedge osteotomy was performed by Dr W. Murray with good healing and excellent function except for a slight residual limp in her gait.

Further studies showed autoantibodies to parathyroid on one occasion (Dr R. Blizzard) and the previously found autoantibodies to adrenal and parietal cells were confirmed by Dr Blizzard and Dr H. Fudenberg. No autoantibodies were found in the patient's parents (Dr Fudenberg). Pituitary growth hormone autoantibodies were negative (Dr W. Hunter). Autoantibodies to human gonadal tissue, including theca and granulosa cells, interstitial cells of testis, as well as to ova and sperm of rabbit and to human trophoblasts were subsequently demonstrated by immunofluorescence (Irvine *et al.*, 1968).

In view of these findings and since gynaecography had demonstrated 'streak' ovaries, exploratory laparotomy was performed. Bilateral 'streak' gonads were found, showing absence of follicles, tubules and no lymphocytic infiltration. Attempts at tissue culture of the streaks and of skin for chromosomal studies were not successful.

When last examined in 1968 and 1969 the patient had started to develop normal breasts and axillary and pubic hair on cyclic oestrogen and progesterone treatment, with regular withdrawal bleeding. Glucose tolerance was normal; there was no evidence of pernicious anaemia; growth hormone and insulin levels were within the normal range both before and after arginine infusion. The patient has attained a height of 153 cm., is mentally alert and fully active except for a minimal limp and is well maintained on vitamin D, calcium carbonate and hydrocortisone as well as on cyclic oestrogen.



## DISCUSSION

When multiple endocrine glands were found to be hypofunctioning it was generally assumed that pituitary failure was present. The more recent recognition of primary 'idiopathic' end-organ deficiency states involving multiple endocrine glands has changed this concept (Christy *et al.*, 1962; Carpenter *et al.*, 1964; Turkington and Lebovitz, 1967). While a possible autoimmune mechanism had been postulated in the past, the recent introduction of modern techniques, especially immunofluorescence, has confirmed the presence of auto-antibodies to both endocrine and non-endocrine tissue in many patients with primary Addison's disease, hypoparathyroidism, thyroiditis, pernicious anaemia and, most recently, with primary ovarian failure (Blizzard *et al.*, 1967; Irvine *et al.*, 1967; Irvine *et al.*, 1968). The presence of such antibodies does not necessarily imply a clinical state of abnormality, but the high frequency of such detectable antibodies may serve as a marker for the possible future development of a deficiency state (Wuepper *et al.*, 1969). Family members who may or may not show clinical endocrine dysfunction likewise may have circulating autoantibodies, suggesting a genetic background for these disorders (Spinner *et al.*, 1968; Wuepper *et al.*, 1967). The finding in our patient with primary hypoparathyroidism and Addison's disease of streak ovaries and other findings suggesting gonadal dysgenesis (Turner's syndrome) raised the possibility of a genetic and autoimmune mechanism rather than a chromosomal abnormality in cases with this disorder. It may be feasible to preserve fertility by the early detection of such autoantibodies to gonadal structures and by the use of immunosuppressive doses of steroids.

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#### DISCUSSION

*Hall:* Do you think this patient has in fact a form of Turner's syndrome with associated autoimmune disease? This would seem to be the most likely explanation in view of the skeletal stigmata. Did you do chromosome studies on the streak ovaries and did you do a skin biopsy? A Turner's mosaic with associated autoimmune disease would be a possibility.

*Kolb:* We tried to culture the streak and we also tried to get a skin biopsy and culture. These were, unfortunately, not successful. It is our feeling that this very well may be an association of Turner's syndrome with autoimmune disease. The fact that there have now been other cases reported where the streaks have had the XX constitution makes us wonder if the streak ovary of Turner's syndrome really is a chromosomal phenomenon or may not have other explanations, such as autoimmunity.

*Irvine:* The association of Turner's syndrome with autoimmunity is very uncommon. I think the literature is a bit confused on this point. Dr W. Price at the Western General Hospital and myself studied the sera of 51 patients with abnormalities of the sex chromosomes, including 21 patients with Turner's syndrome. We were unable to find any patients with antibodies to adrenal, to other steroid-producing cells or to ova in these groups. In fact, we were unable to confirm that patients with chromosome abnormalities do indeed have any increased incidence of autoimmunity (Price, W. H. and Irvine, W. J. 1969. *Clin. expl. Immun.* **4**, 365). So there is something very exceptional about Dr Kolb's patient.

*Hartog:* Is this instance of fractures in Turner's syndrome unusual? Secondly, do you think that prednisolone treatment, by analogy say with pernicious anaemia or autoimmune thyroiditis, might be successful in partially reversing the autoimmune process? Would its use be possibly justifiable in terms of trying to promote pregnancy?

*Kolb:* We have not seen fractures but we have seen similar hip

## DISCUSSION

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deformities and osteoporosis in Turner's syndrome. The fact that she fractured her hips may be related to her convulsive tendency.

Whether or not it would be safe to use large amounts of corticosteroids remains an open question. In this instance, having Addison's disease and osteoporosis, we had no choice but to use physiological doses of corticosteroids. I am not sure whether this had anything to do with the disappearance of her goitre. I do not think the use of pharmacologic doses of corticosteroids would have made any difference in this youngster, because I believe her autoimmunization probably goes back to intrauterine life. I doubt that we could have preserved fertility by enhancing her corticosteroid therapy.

CHAPTER V

CHAPTER V

AUTOIMMUNITY IN DIABETES MELLITUS

"Gastric Antibodies in Diabetes Mellitus"  
(1963) Lancet, ii, 838-839.

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V : 1-4

"The Incidence of Thyroid and Gastric  
Autoimmunity in Patients with Diabetes  
Mellitus" (1969) Clinical Science, 37, 570.

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"Thyroid and Gastric Autoimmunity in Patients  
with Diabetes Mellitus" (1970) ~~Submitted~~ *Accepted*  
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**GASTRIC ANTIBODIES IN DIABETES MELLITUS**

**W. J. Irvine and S. H. Davies**

**Lancet (1963), ii, 838-839.**

*Letter to the Editor**reprinted from THE LANCET, October 19, 1963, pp. 838-839***GASTRIC ANTIBODIES IN DIABETES MELLITUS**

SIR,—For some time now we have been testing the sera of a large number of patients for the presence of gastric parietal-cell antibody, in an attempt to assess the clinical significance of a positive finding. It was therefore with great interest that we read the article by Dr. Moore and Dr. Neilson (Sept. 28).

Of the sera we have analysed to date, 170 were from patients with proven diabetes mellitus but in whom there was no clinical evidence of pernicious anaemia or of thyroid disease. All these diabetic sera have been examined for gastric parietal-cell antibody both by the indirect Coons fluorescent-antibody technique<sup>1 2</sup> and by the method of complement fixation. The details of the complement-fixation method used in our study have been given elsewhere<sup>3</sup> and differ in some respects from those adopted by Moore and Neilson. In our experience the complement-fixation test varies in reproducibility by plus or minus one titre when doubling dilutions are used. One of the main reasons for this is the difficulty of assessing 50% hæmolysis in the indicator system. A positive gastric complement fixation titre of 1:4 was therefore considered to be valid only if a positive result was obtained on at least 3 out of 4 consecutive testings or if confirmed by unequivocal staining of parietal cells by the fluorescent-antibody method. The fluorescent-antibody method has been shown to be a more sensitive indicator for the detection of gastric parietal-cell antibody than the method of complement fixation and has the advantage of having a clearer and more reproducible end-point; it also overcomes many of the problems of tissue specificity.

When our diabetic patients are classified into those who were diagnosed before the age of forty years (early onset) and those whose disease became manifest after the age of forty years (late onset) the results are as shown in table 1. These patients have been analysed according to their sex and their present age and the serological findings compared with a similar analysis of the sera of 629 blood-donors. Gastric parietal-cell

1. Taylor, K. B., Roitt, I. M., Doniach, D., Couchman, K. G., Shapland, C. *Brit. med. J.* 1962, ii, 1347.
2. Irvine, W. J. *Quart. J. exp. Physiol.* 1963, 48, 427.
3. Irvine, W. J., Davies, S. H., Delamore, I. W., Williams, A. W. *Brit. med. J.* 1962, ii, 454.

TABLE I—ANTIBODIES IN SERA OF DIABETICS

Age at onset of diabetes	Sex	Total number of patients	Present age less than 40 years	Antibodies							A.N.F.
				Gastric		Thyroid			C.F. and/or T.C.H.		
				I.C.	C.F.	C.F.	T.C.H.				
Early (<40 years)	F	26	14	3	2	1	1	—	1	2	1
	M	34	20	4	3	—	4	3	5	—	—
	Total	60 <sup>1</sup>	34	12%	8%	3%	8%	9%	5%	10%	9%
Late (>40 years)	F	63		9	8		11	12	18	1	
	M	40		5	4		—	—	—	1	
	Total	103 <sup>2</sup>		14%	12%		11%	12%	17%		2%

1. Average present age 36.2 years. 2. Average present age 65.1 years.

I.C., indirect Coombs fluorescent-antibody technique.

C.F., complement fixation test using 2.0 M.H.B. of complement, 50% hemolysis.

T.C.H., tanned cell hemagglutination test for antibody to thyroglobulin.

A.N.F., antinuclear factor(s).

TABLE II—ANTIBODIES AND FAMILY HISTORY IN DIABETICS

No. of patients	Family history	Gastric I.C.	Thyroid C.F.T. and/or T.C.H.	A.N.F.
5	Pernicious anaemia	..	1	1
5	Pernicious anaemia	..	1	1
10	Diabetes	1	2	1
3	Thyroid disease	..	3	..
3	Thyroid disease	..	3	..
26	Diabetes	1	..	..
26	Diabetes	4	4	1
49	Any of the above	6	10	3
54	None of the above	7	6	1

antibody was detected by the fluorescent-antibody method in 3% of 140 female and in 1% of 168 male blood-donors under the age of forty years, and in 10% of 164 female and in 6% of 157 male blood-donors over the age of 40. On this evidence we cannot confirm the striking incidence of gastric parietal-cell antibody in the early-onset type of diabetes described by Moore and Neilson. We assume from the low incidence of antibodies in the matched controls that their patients in the early-onset group are at present of early adult age. When the incidence of gastric parietal-cell antibody is analysed by decades using the present age of the diabetic patients and of the blood-donors, then we find that the diabetic who is presently middle-aged (40-60 years) has a higher incidence of both gastric and thyroid antibodies than the blood-donors. This difference exists but is not pronounced.

A careful family history of addisonian pernicious anaemia, thyroid disease, and diabetes in the blood relatives was taken in 103 of the 170 diabetic patients, and the findings are correlated with the patient's serology in table II. While there is a higher incidence of thyroid antibodies and of anti-nuclear factor in the patients with a positive family history, the present figures do not show this to be so with regard to antibody to gastric parietal cells. However, in a study of a larger series of relatives of patients with addisonian pernicious anaemia (but who may not have diabetes) we do find a definitely increased incidence of gastric parietal-cell antibody in their sera. A detailed clinico-immunological study of a much larger number of patients with diabetes mellitus may demonstrate that these hereditary factors and the age of the patient at the time the serum is tested are more directly relevant to understanding the incidence of gastric parietal-cell antibody in diabetes mellitus than the age of the patient when the diabetes was first diagnosed.

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THYROID AND GASTRIC AUTOIMMUNITY IN PATIENTS WITH  
DIABETES MELLITUS

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(1969) *Clinical Science*, 37, 570

# 17. THE INCIDENCE OF THYROID AND GASTRIC AUTOIMMUNITY IN PATIENTS WITH DIABETES MELLITUS

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The organ-specific autoimmune thyroid diseases and pernicious anaemia are alleged to occur more frequently, in diabetic subjects. In the present study, the incidence of antibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm was found to be significantly increased ( $P < 0.001$ ) in the sera of 883 diabetics compared to 866 control subjects when analysed by age and sex. There was no clear relationship between the incidence of these antibodies with the duration of diabetes, but in patients over the age of 40 years at the time of study the incidence was significantly higher in those who were insulin dependent ( $P < 0.005$ ). Within the insulin dependent group there was no correlation with the age of onset of the diabetes. There was no overall difference in the incidence of antibody to thyroglobulin in the diabetics compared to controls.

Intrinsic factor antibody I was found more commonly in female diabetics over the age of 40 years than in female controls of comparable age. Of nine such patients with intrinsic factor antibody who were available for study, there was evidence of achlorhydria in all, malabsorption of vitamin B<sub>12</sub> without anaemia in three, and of adequate vitamin B<sub>12</sub> absorption in four.

There would appear to be some disturbance of the immunological system in relation to diabetes with respect to the formation of autoantibodies and the occurrence of organ-specific autoimmune disease.

THYROID AND GASTRIC AUTOIMMUNITY IN PATIENTS  
WITH DIABETES MELLITUS

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### SUMMARY

The organ-specific autoimmune thyroid diseases and pernicious anaemia are alleged to occur more frequently in diabetic subjects. In the present study, the incidence of antibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm was found to be significantly increased ( $p < 0.001$ ) in the sera of 1054 diabetics without clinical thyroid disease or pernicious anaemia compared to 871 control subjects when analysed by age and sex. There was no clear relationship between the incidence of these antibodies with the duration of diabetes, but in patients over the age of 40 years at the time of study the incidence was significantly higher in those who were insulin-dependent ( $p < 0.001$ ). Within the insulin-dependent group there was no correlation with the age of onset of the diabetes. The incidence of antibody to thyroglobulin in the diabetics compared to controls was much less pronounced than was the incidence of antibody to thyroid cytoplasm and to gastric parietal cell cytoplasm.

Intrinsic factor antibody 1 was found more commonly in female diabetics over the age of 40 years than in female controls of comparable age ( $p < 0.005$ ). Of 9 such patients with intrinsic factor antibody who were available for study there was evidence of achlorhydria in all, latent pernicious anaemia (with malabsorption of vitamin B<sub>12</sub>) in 6 and of adequate vitamin B<sub>12</sub> absorption in 3.

There would appear to be a disorder of the immunological system in relation to insulin-dependent diabetes with respect to the formation of autoantibodies and the occurrence of organ-specific autoimmune disease.



## INTRODUCTION

On account of the apparent clinical association between diabetes mellitus and diseases characterised by the occurrence of organ-specific autoimmunity,<sup>1, 2, 3, 4</sup> an extensive study was undertaken to determine the prevalence of gastric and thyroid autoimmunity in diabetics compared to age and sex matched controls and to find out if there is any difference in this respect between insulin-dependent diabetics and insulin-independent diabetics. It was also hoped that these studies would yield further information on the prevalence of pernicious anaemia in its latent form in diabetics.

## SUBJECTS STUDIED

The patients with diabetes mellitus consisted of 521 females and 533 males. Care was taken to exclude any diabetic patient with clinic evidence or personal history of thyroid disease or pernicious anaemia. The patients were obtained by random selection among those attending the Diabetic Out-Patient Department until such time as the numbers obtained in each particular age and sex group were considered adequate or until further numbers seemed unlikely to be readily forthcoming. The age of symptomatic onset and the duration of the diabetes was noted for each patient. In addition information regarding a family history for diabetes, thyroid disease and pernicious anaemia in the first degree relatives was obtained from 913 of the total 1,054 diabetics.

The control subjects consisted of 443 females and 428 males. They were mainly blood donors, but hospital controls were included at the extremes of the age range. The hospital controls were not known to be suffering from clinical diseases associated with autoimmunity.

The age and sex distribution of the diabetic and control subjects (a total of 1,925 subjects) is shown in Fig. 1. Table 1 shows the analysis of the diabetics according to the treatment they required; diet alone, oral

## AGE AND SEX DISTRIBUTION OF DIABETICS AND CONTROLS

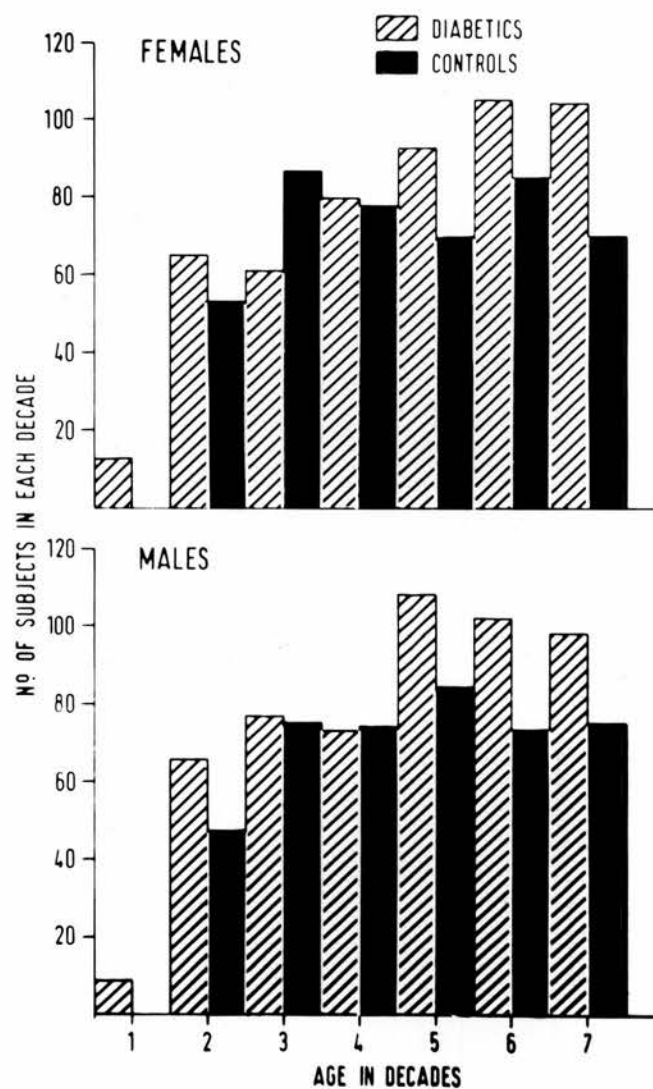


Fig. 1

Age and sex distribution of the total 1054 diabetics and of the 871 controls included in the present study.

TABLE 1

ANALYSIS OF THE 1054 DIABETIC PATIENTS  
ACCORDING TO THE TREATMENT THEY REQUIRED

	<u>Age at time of serological study</u>			
	<u>0-39 years</u>		<u>40-69 years</u>	
	<u>Females</u>	<u>Males</u>	<u>Females</u>	<u>Males</u>
Diet	21) ) 35	9) ) 21	66) ) 153	63) ) 152
Oral hypoglycaemic agents	14)	12)	87)	89)
Insulin	184	204	149	156
TOTAL NUMBER	219	225	302	308

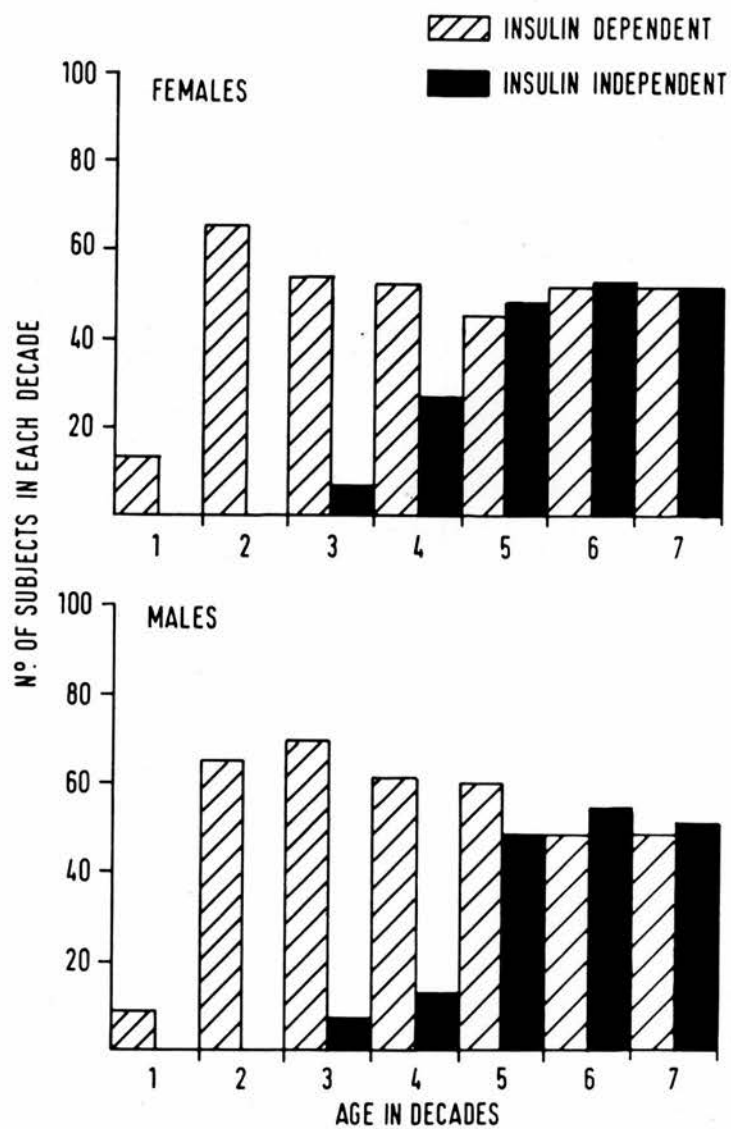


Fig. 2

The age and sex distribution of insulin-dependent and insulin-independent diabetics in the total of 1054 diabetics.



hypoglycaemic agents, or insulin. Diabetics controlled by diet alone or oral hypoglycaemic agents are referred to as insulin-independent; those requiring insulin are referred to as insulin-dependent. Fig. 2 shows the age and sex distribution of the insulin-dependent and of the insulin-independent diabetics. There were an approximately even number of patients in the two groups of diabetics studied in the 5-7th decades (aged 40-69 years) and they were equally distributed in these three decades. The number of insulin-independent diabetics in the younger age group (0-39 years) was small and these patients were mainly in the fourth decade.

## METHODS

Sera were stored at  $-20^{\circ}\text{C}$  in small volumes, and tested in batches for the following antibodies:-

Antibody to thyroglobulin, using the tanned cell haemagglutination test<sup>6</sup> with the reagents as supplied by Burroughs Wellcome, Beckenham, Kent, England. The test was done in serum dilutions of 1:5, 1:25, 1:2,500 and 1:25,000. A titre of greater or equal to 1:25 was taken as being significant.

Antibody to thyroid cytoplasm, using the indirect immunofluorescence test with snap-frozen air-dried sections of human thyroid obtained at partial thyroidectomy for thyrotoxicosis.

Antibody to gastric parietal cell cytoplasm, using the indirect immunofluorescence test with snap-frozen air-dried sections of gastric mucosa obtained from the body of human stomach at partial gastrectomy.

As a check for tissue specificity each serum was tested by the indirect immunofluorescence method for reactivity with rat kidney, all three tissues (thyroid, stomach and kidney) being mounted together on the same slide. The details of the reagents and procedure were as described previously.<sup>3,7</sup>

Sera giving positive reactions with rat kidney (notably staining of the mitochondria in the renal tubules) were regarded as being negative for thyroid cytoplasm and gastric parietal cell antibodies, because of the non-tissue-specific nature of the mitochondrial antibodies.<sup>8</sup> The presence or absence of nuclear staining was also noted in all sections.

Antibody to intrinsic factor. The sera of all the female diabetics and of female controls aged 30 years and older were tested for intrinsic factor antibody I by radioimmunoassay using albumin-coated charcoal as previously described.<sup>9</sup> A titre of greater or equal to 5.0 units/ml serum was regarded as positive.

The incidence of antibodies in the sera of diabetics compared to the controls was analysed statistically using the Chi<sup>2</sup> test and Yates' correction using Fisher's "exact test" when applicable. Statistical significance was assumed when  $p < 0.05$ .

Diabetic patients whose sera was positive for intrinsic factor antibody I were studied with regard to their gastric function and vitamin B<sub>12</sub> metabolism. The gastric juice secreted during the hour following intramuscular pentagastrin (6µg/Kg body weight) was aspirated and measured without delay for total content of HCl using a pH meter and an automatic titrator (type TTT 1c "Radiometer", Copenhagen) with 1.0N NaOH. Achlorhydria was defined as a pH of not less than 6.0 during the post-stimulation hour. The gastric juice secreted during the post-stimulation hour was also measured for total intrinsic factor content using radioimmunoassay as previously described.<sup>9</sup> An intrinsic factor secretion during the post-stimulation hour of greater than 200 units is considered incompatible with Addisonian pernicious anaemia, most patients with frank or latent pernicious anaemia having an intrinsic factor secretion of less than 100 units in the post-stimulation hour.

The serum vitamin B<sub>12</sub> level was determined microbiologically.<sup>10</sup> Bone marrow smears were studied in the ordinary manner. Intestinal absorption of vitamin B<sub>12</sub> was determined by the Schilling test using 0.5µGm <sup>58</sup>Co-vitamin B<sub>12</sub> with specific activity 1µ Ci/µGm followed 1 hour later by 1000µGm Cytamen. A 24 hour urinary secretion of more



than 12.5% of the oral dose was taken as normal, 7.5-12.5% as being equivocal and less than 7.5% as indicative of malabsorption of vitamin B<sub>12</sub>. All patients included in the study had adequate renal function. When the Schilling test was repeated with intrinsic factor, an oral dose of at least 1000 units of hog intrinsic factor was used.<sup>11</sup>

Intestinal absorption of vitamin B<sub>12</sub> was also studied by a total body counting technique.<sup>12</sup> A total body count at seven days of less than 21% of the initial dose is indicative of malabsorption of vitamin B<sub>12</sub>, 21-25% equivocal and greater than 25% is indicative of adequate vitamin B<sub>12</sub> absorption. The total body count estimations of vitamin B<sub>12</sub> absorption were also done with pentagastrin (6µG/kg) given 10 minutes before the oral dose <sup>58</sup>Co-vitamin B<sub>12</sub>. This amounts to a repeat test, as previous studies have demonstrated that pentagastrin stimulation makes no consistent difference to the total body count results in vitamin B<sub>12</sub> absorption.



## RESULTS

Fig. 3 shows the percentage of the diabetics and of the controls whose sera were positive for antibodies to thyroglobulin (tanned cell titre greater or equal to 1:25), to thyroid cytoplasm and to gastric parietal cell cytoplasm. Antinuclear antibodies and mitochondrial antibodies were only detected in 1% of the diabetics and of the controls.

In the female control subjects a rising incidence of antibodies to thyroglobulin, to thyroid cytoplasm and to gastric parietal cell cytoplasm was observed with age. Although the incidence of antibodies to thyroglobulin was significantly higher in the diabetics compared to the controls when the total numbers of diabetics and controls were analysed ( $p < 0.01$ ), the  $p$  values corresponding to the different age groups and sexes either did not reach significance or did so only marginally. In contrast, the increased incidences of antibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm respectively were statistically significant in diabetics compared to the controls when all subjects were considered ( $p < 0.001$ ) and when sub-groups of subjects according to sex and to age (<40 years or  $\geq 40$  years) were considered ( $p < 0.005$  in all sub-groups).

In Table 2 the incidence of thyroid and of gastric antibodies in the sera of insulin-dependent and of insulin-independent diabetics is compared with that in controls of comparable age and sex. In insulin-dependent diabetics the increased incidence of antibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm was highly significant in both males and females, aged 10-39 years as well as in those aged 40-69 years. The incidence of antibody to thyroglobulin in the insulin-dependent diabetics compared to controls was not significant in these sub-groups except in insulin-dependent males aged 40-69 years. In the insulin-independent diabetics, the difference in the incidence of autoantibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm compared to controls

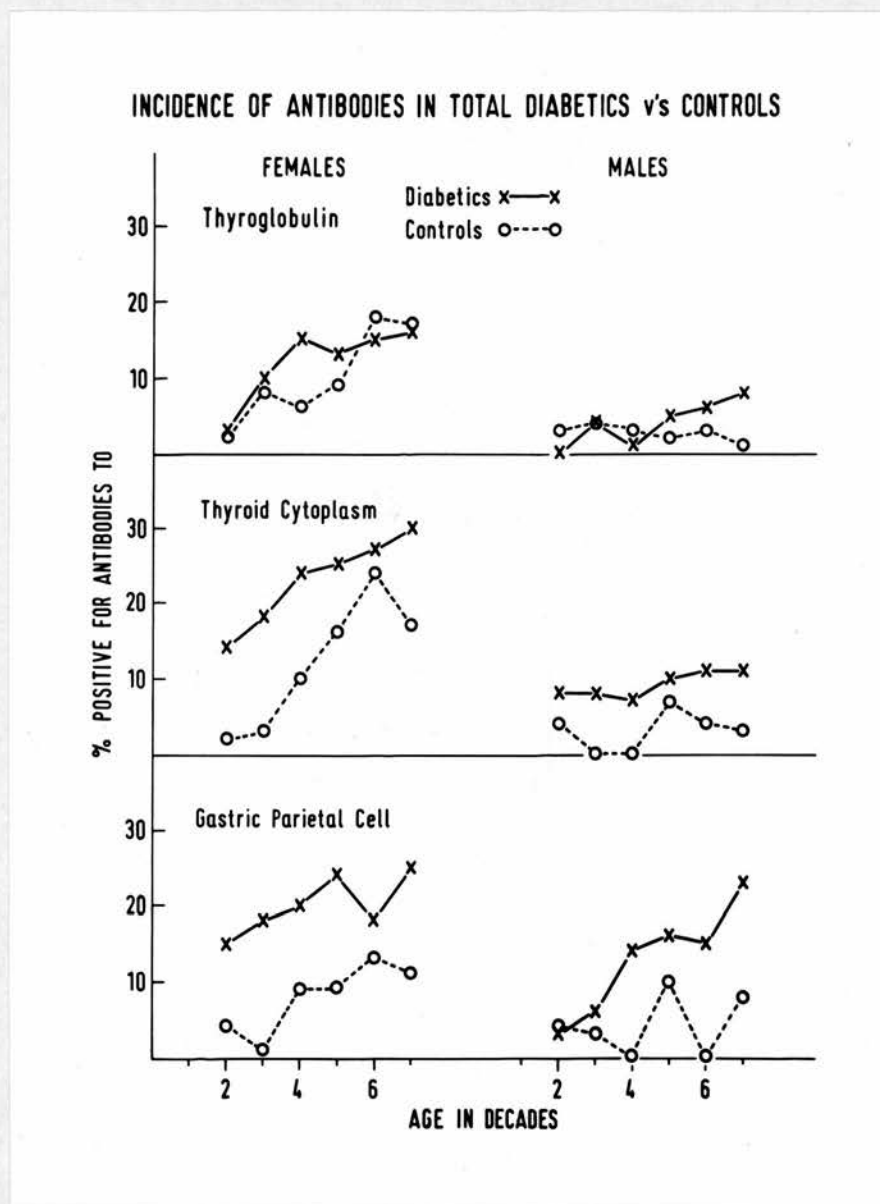


Fig. 3

Percentage incidence of autoantibodies specific for thyroglobulin, thyroid cytoplasm and gastric parietal cell cytoplasm, respectively, in the total diabetics (except those aged 0-9 years) and in the controls according to sex and age in decades.

TABLE 2

INCIDENCE OF POSITIVE TESTS FOR ANTIBODIES TO THYROGLOBULIN, THYROID CYTOPLASM  
AND GASTRIC PARIETAL CELL CYTOPLASM IN DIABETIC PATIENTS COMPARED TO CONTROLS

	<u>CONTROLS</u>		<u>DIABETICS</u>			
	<u>Female</u>	<u>Male</u>	<u>Insulin-dependent</u>		<u>Insulin-independent</u>	
			<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>
<u>Subjects aged 10-39 years</u>						
No. positive for antibody to:						
Thyroglobulin	13	5	15(N.S.)*	4(N.S.)	5(N.S.)	2(N.S.)
Thyroid cytoplasm	12	2	37(<0.001)	13(<0.001)	2(N.S.)	3(<0.014)
Gastric parietal cell cytoplasm	10	4	33(<0.001)	15(<0.005)	4(N.S.)	2(N.S.)
Total No. of subjects	218	196	171	195	35	21
<u>Subjects aged 40-69 years</u>						
No. positive for antibody to:						
Thyroglobulin	33	5	25(N.S.)	11(<0.01)	20(N.S.)	8(N.S.)
Thyroid cytoplasm	43	11	46(<0.001)	22(<0.001)	36(N.S.)	11(N.S.)
Gastric parietal cell cytoplasm	25	14	40(<0.001)	37(<0.001)	27(<0.05)	18(<0.025)
Total No. of subjects	225	232	149	156	153	152

( ) \* statistical significance of the incidence of the antibodies in sub groups of diabetics compared to that in controls matched for age and sex.

Using  $X^2$  test and Yates' correction, Fisher's exact test was applied when appropriate, significance limit  $p < 0.05$ .

N.S. = Not significant.

was not so striking as in the case of the insulin-dependent diabetics. Indeed, in insulin-independent diabetics the incidence of antibodies to thyroid cytoplasm and to gastric parietal cell cytoplasm only reached statistical significance in three of the eight sub-groups indicated in Table 2. There was no difference in the incidence of thyroglobulin antibodies in the different sub-groups of insulin-independent diabetics compared to controls.

When insulin-dependent and insulin-independent diabetics were directly compared, the insulin-dependent diabetics were shown to have a significantly higher incidence of antibodies to thyroid cytoplasm and/or to gastric parietal cell cytoplasm with p values of  $\leq 0.01$  for females,  $\leq 0.001$  for males and  $\leq 0.001$  when both sexes were considered together.

There was no significant difference in the incidence of antibodies to thyroid cytoplasm or to gastric parietal cell cytoplasm in patients who had had insulin treatment for diabetes for 5 years or longer compared to the incidence in those who had had insulin for a shorter period. Nor was there any difference in those who had had insulin for 10 years or more compared to a shorter period in an analysis of 692 insulin-dependent diabetics. Likewise in insulin-dependent diabetics there was no significant difference in the incidence of these antibodies in patients whose diabetes developed early ( at 0-39 years) or later ( at age 40 years or older). This would suggest that the duration of diabetes may not be an important factor in the production of thyroid or gastric autoantibodies.

Out of the 913 diabetics included in the family history study, 25% gave a positive family history for diabetes, 9% for thyroid disease and 3% for pernicious anaemia in first degree relatives. A family history of diabetes showed no correlation with the incidence of thyroid or of gastric autoantibodies in insulin-dependent, insulin-independent or total diabetics. When insulin-dependent and insulin-independent diabetics of all ages and both sexes were considered, an increased incidence of antibodies to thyroid cytoplasm and/or to gastric parietal cell cytoplasm was noted in



those with a first degree family history of thyroid disease ( $p < 0.03$ ) and of pernicious anaemia ( $p < 0.05$ ) compared to those without such a family history.

The incidence of intrinsic factor antibody I in the sera of female diabetics is shown in Table 3. This antibody was not detected in diabetics in the first 3 decades, but was present in the serum of 13 of the 380 diabetics aged 30-69 years. The age of the youngest diabetic with intrinsic factor antibody in the serum in this series was 39 years. Only one out of the 296 female controls subjects aged 30-69 years was positive for intrinsic factor antibody: a subject aged 67 years. The increased incidence of intrinsic factor antibodies in middle aged and elderly female diabetics was statistically higher than in age and sex matched control subjects ( $p < 0.005$ ). As shown in Table 4, eight of the thirteen diabetics positive for intrinsic factor antibody were insulin-dependent and 5 were insulin-independent. The incidence of intrinsic factor antibodies would therefore appear to be related to age rather than the type of diabetes.

Table 4 also summarises the results of tests of gastric function and of vitamin B<sub>12</sub> status carried out on the 10 diabetics whose sera were positive for intrinsic factor antibodies and in whom it was possible to do at least some of these analyses. All those tested for acid secretion had achlorhydria, using pentagastrin stimulation and aspiration of gastric juice in 8 patients and the azuresin test ('Diagnex Blue', Squibb) in one patient. The secretion of intrinsic factor into the gastric juice in the post-pentagastrin hour was found to be less than 100 units in 7 patients and greater than this but less than 200 units in one patient. As indicated in Table 4 difficulty in aspiration of the gastric juice was encountered in one patient. In five patients (nos. 1, 2, 3, 4 & 6) the results of the Schilling tests of vitamin B<sub>12</sub> absorption without and with oral intrinsic factor were typical of Addisonian pernicious anaemia, although the serum vitamin B<sub>12</sub>

TABLE 3: INTRINSIC FACTOR ANTIBODY I IN FEMALE DIABETICS NOT PREVIOUSLY KNOWN TO HAVE PERNICIOUS ANAEMIA COMPARED TO CONTROL SUBJECTS

<u>Age (in years)</u>	<u>FEMALE DIABETICS</u>		<u>FEMALE CONTROLS</u>	
	<u>Total No. Tested</u>	<u># I. F. Antibody pos.</u>	<u>Total No. Tested</u>	<u># I. F. Antibody pos.</u>
0-9	13	nil	-*	-
10-19	64	nil	-	-
20-29	61	nil	-	-
<hr/>				
30-39	80 )	1	76	nil
40-49	92 )	1	70	nil
50-59	105 )	2	84	nil
60-69	103 )	9	66	1
	380 )	13	296	1

\*- = not tested

# I. F. = intrinsic factor

No	Age	Treatment required for diabetes	I.F. antibody titre in (units)	Gastric Analysis (post-pentagastrin hr.)	Vitamin B12 Absorption				Marrow	Conclusion		
					Schilling test (%)	Total body Count		Serum Vit. B12				
						without oral I.F.	with oral I.F.				-P	+P
			(units)	acid	I.F. (units)					(pg/ml)		
1	51	insulin	90	achlorhydria	nil	0.6	15.0	nil	5	112	normoblastic	latent P.A.
2	67	insulin	33	achlorhydria	nil	1.8	14.1	8	28	159	normoblastic	latent P.A.
3	65	oral hypoglyc.	38	achlorhydria	2	1.7	23.2	11	9	50	normoblastic	latent P.A.
4	62	insulin	6	achlorhydria	86	1.7	27.8	13	4	563	normoblastic	latent P.A.
5	60	oral hypoglyc.	7	achlorhydria	39	-	-	-	21	113	-	latent P.A.
6	69	insulin	5	-	-	1.2	15.3	13	12	50	normoblastic	latent P.A.
7	65	insulin	50	achlorhydria	9	14.0	-	22	24	178	normoblastic	borderline B12 absorption
8	39	insulin	12	achlorhydria (poor colln.)	nil	14.0	-	45	51	303	-	adequate B12 absorption
9	59	insulin	96	achlorhydria	167	10.0	-	26	78	174	normoblastic	adequate B12 absorption
10	63	insulin	138	achlorhydria*	-	-	-	-	-	-	-	-
11	41	oral hypoglyc.	6	-	-	-	-	-	-	-	-	-
12	64	diet	38	-	-	-	-	-	-	-	-	-
13	63	oral hypoglyc.	12	-	-	-	-	-	-	-	-	-

- = Not tested

-P = without prior. pentagastrin stimulation

+P = with prior pentagastrin stimulation

I.F. = intrinsic factor

\*by Diagnex test

\*by Diagnex test

I.F. = intrinsic factor

-P = without prior pentagastrin stimulation  
+P = with prior pentagastrin stimulation

- = Not tested

TABLE 4: VITAMIN B12 STATUS IN FEMALE DIABETIC PATIENTS WITH INTRINSIC FACTOR ANTIBODY I IN THE SERUM



levels were in the equivocal zone in two of those patients and normal in a third. The bone marrow was normoblastic in all five of these patients before any parental vitamin B<sub>12</sub> was given. These patients were therefore considered to have latent pernicious anaemia; that is to say, they had abnormal vitamin B<sub>12</sub> absorption due to lack of intrinsic factor secretion but the body stores of vitamin B<sub>12</sub> were not sufficiently depleted to produce a megaloblastic anaemia.<sup>12.</sup>

Patient no. 5 was also considered to have latent pernicious anaemia in view of the achlorhydria and minimal intrinsic factor secretion and evidence of malabsorption of vitamin B<sub>12</sub> by the total body counting method, together with a low serum vitamin B<sub>12</sub> level. In patient no. 7 the intrinsic factor secretion measured by radioimmunoassay was very low (9 units in the post pentagastrin hour) and the total body count results for vitamin B<sub>12</sub> absorption were equivocal, but the Schilling test was normal and the serum vitamin B<sub>12</sub> level was at the lower limit of normal. Patients nos. 8 and 9 had adequate B<sub>12</sub> absorption in spite of the presence of intrinsic factor antibodies in the serum. No information could be obtained regarding the vitamin B<sub>12</sub> status of patients nos. 10-13.



## DISCUSSION

Landing et al<sup>13</sup> found thyroid antibodies using the indirect immunofluorescence technique in 13.3% of 83 juvenile onset diabetics under the age of 16 years, while the incidence in 178 euthyroid non-diabetic children was 1.1%.<sup>14</sup> Moore and Neilson<sup>15</sup> studied serum from 83 diabetics and concluded that gastric complement-fixing antibodies were significantly more common in early-onset type diabetes.

In their series of 317 diabetics and 624 controls, Simkins<sup>16</sup> found evidence that antibodies to thyroglobulin occur more frequently in diabetics without clinical thyroid disease than in control subjects ( $p < 0.01$ ). While we agree with this overall statistic, the increased incidence of thyroglobulin antibodies is not nearly so impressive as that for antibodies to thyroid cytoplasm, gastric parietal cell cytoplasm and intrinsic factor.

Whereas in 200 insulin-dependent diabetics aged 40 years or less Ungar et al<sup>17</sup> found a significantly higher incidence of parietal cell antibodies in females but not in males, we found these antibodies to be significantly increased in both sexes in this age group. We agree with these workers that both male and female insulin-dependent diabetics over 40 years have a significantly higher incidence of parietal cell antibodies than do controls.

In relation to insulin-independent diabetes, when Ungar's and the present series are considered together it is doubtful if gastric parietal cell antibodies are increased in any sub-group. From the present study it is also probable that the incidence of antibodies to thyroid cytoplasm is not significantly higher in insulin-independent diabetics of any sub-group by age or sex. The p value of  $< 0.014$  for antibodies to thyroid cytoplasm in insulin-independent diabetic males aged 10-39 years compared to controls may not be sustained if it were possible to study a larger number of patients in this sub-group. Neither Ungar et al<sup>17</sup> nor Simkins<sup>16</sup> studied antibodies to thyroid cytoplasm in their series. The increased incidence of antibodies

to thyroid cytoplasm and to gastric parietal cell cytoplasm in insulin-dependent compared with insulin-independent diabetics does not hold for antibodies to thyroglobulin.

The inheritance of diabetes mellitus is probably complex and former single gene hypotheses such as simple recessive inheritance with reduced penetrance<sup>18</sup> have been mainly abandoned in favour of a multifactorial hypothesis, which involves the additive effects of several genes.<sup>19</sup> Such an hypothesis would explain the differences between juvenile-onset (insulin-dependent) diabetes where genetic factors are stronger and adult-onset (insulin-independent) diabetes where genetic influences are less strong and where environmental factors such as obesity, pregnancy, infection and other stress may determine the onset of the overt disease.

With regard to intrinsic factor antibody I, the findings in the present study agree with those of Ungar et al<sup>17</sup> in that these antibodies were only found in middle aged and elderly diabetics, but differ in that they occurred in an equal number of insulin-dependent and insulin-independent diabetics. In the present study achlorhydria was demonstrated in all the intrinsic factor antibody positive patients tested and this was the case in all but one in the series of Ungar et al,<sup>17</sup> which is in keeping with the general finding that intrinsic factor antibodies in the serum show a high correlation with advanced atrophic gastritis.<sup>20, 21, 22</sup> However, a surprisingly high number of diabetic patients with intrinsic factor antibody in the serum (3 out of 9 in the present series and 4 out of 8 in the series reported by Ungar and her colleagues) had adequate or at least borderline vitamin B<sub>12</sub> absorption. On the other hand, six out of our nine patients whose sera were positive for intrinsic factor antibodies and who had vitamin B<sub>12</sub> absorption studies done had latent pernicious anaemia.

Ungar et al<sup>17</sup> found the prevalence of latent pernicious anaemia in diabetes (with or without intrinsic factor antibodies in the serum) to be



4%, but this incidence must clearly depend on the age and sex distribution of the population of diabetics studied. In the present series 3.4% of female diabetics aged 40-69 years had intrinsic factor antibodies in the serum. As only 50-60% of patients with frank pernicious anaemia or latent pernicious anaemia are intrinsic factor antibody positive<sup>23, 21, 24, 25</sup> and because a minority of intrinsic factor antibody patients have adequate vitamin B<sub>12</sub> absorption, it can be estimated that approximately 5% of female diabetics aged 40 years or more (insulin-dependent or insulin-independent) have latent pernicious anaemia. A further percentage will have frank pernicious anaemia, but these patients were excluded from the present study and no statistic can be given.

In a review of the literature Chanarin<sup>26</sup> states that the frequency of pernicious anaemia in diabetes mellitus is 3.9 per 1,000 which, he states, is the expected frequency of pernicious anaemia after the age of 40 years in the general population. Conversely, he states that the incidence of diabetes in pernicious anaemia is 2.4%, which is not greatly different from the incidence of diabetes mellitus in the general population where it has been reported as 1.3%<sup>27</sup> and 1.7%<sup>28</sup>. The present studies, together with those of Ungar et al,<sup>17</sup> which unequivocally indicate that intrinsic factor antibodies occur more commonly in middle aged and elderly diabetics than in controls, would question the validity of previous clinical studies. There can be little doubt that pernicious anaemia, at least in the latent form, is more common in the middle aged and elderly diabetics than in control subjects. The discrepancies are probably due to the inadequate diagnostic criteria for pernicious anaemia in some of the large series of patients with diabetes mellitus included in Chanarin's review.

When latent pernicious anaemia is defined as in the present paper, it would seem inevitable that such patients would eventually proceed to frank pernicious anaemia when their body stores of vitamin B<sub>12</sub> were

depleted. This progression need not occur when the intrinsic factor secretion, although reduced, is still adequate to maintain vitamin B<sub>12</sub> absorption.

An incidence of 5% latent pernicious anaemia in middle aged to elderly female diabetics is of considerable clinical importance and it would be reasonable to screen all diabetics of this sex and age group for intrinsic factor antibodies and to check their serum vitamin B<sub>12</sub> levels. Ungar et al<sup>17</sup> noted that only 1 of their 8 intrinsic factor positive diabetics was male. It is uncertain whether insulin-dependent diabetics are at greater risk than insulin-independent diabetics for latent pernicious anaemia.

While circulating antibody to insulin may occur in diabetic patients treated with bovine insulin,<sup>29</sup> insulin antibodies are usually not detected in persons not receiving insulin. The work of Mancini<sup>30</sup> claiming the occurrence of antibodies to pancreas in untreated diabetics and of Penchev et al<sup>31</sup> claiming that precipitating antibody to insulin can be found in 20% of untreated diabetics is still to be confirmed.

Lymphocytic infiltration of the islets of Langerhans has been observed in a few human diabetics prior to insulin therapy, particularly in the acute-onset juvenile type.<sup>32</sup> Lee et al<sup>33</sup> claim that this lesion strongly resembles that of the experimental diabetes that they were able to produce in New Zealand white rabbits by immunization with bovine insulin in Freund's adjuvant. Hyperglycaemia or diabetes mellitus developed in some of the immunized animals. The pancreas of the diabetic animals showed lymphocytic infiltration of the islets of Langerhans, while rabbits with insulin antibody but without islet lymphocytes remained normoglycaemic. This would suggest that if immune mechanisms play some part in the pathogenesis of diabetes mellitus, delayed hypersensitivity may be of greater significance than humoral antibodies.

The evidence in the present paper would suggest that insulin-



dependent diabetes is more closely associated with disturbed immunity (whether specific for the pancreas or not) than is the case in insulin-independent diabetes. Further work on the role of immunity in the pathogenesis of insulin-dependent diabetes is clearly required.

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CHAPTER VI

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AUTOIMMUNITY AND LUNG DISEASE

"Idiopathic Diffuse Interstitial Lung Disease"  
(1965) Amer. rev. resp. Dis., 92, 939-948.

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IDIOPATHIC DIFFUSE INTERSTITIAL LUNG DISEASE

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## IDIOPATHIC DIFFUSE INTERSTITIAL LUNG DISEASE<sup>1</sup>

### A Review of 42 Cases

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AND MARGARET A. J. MOFFAT

(Received for publication May 6, 1965)

#### INTRODUCTION

Although the first recognized cases of a form of interstitial lung disease were described under the title of "acute diffuse interstitial fibrosis of lung," by Hamman and Rich in 1933 (1), there is at least one earlier account of similar pathologic findings in the lungs of patients with progressive pulmonary disease (2). Because of variations in the course of the disease and in the pathologic features, a number of different terms have been used to describe individual cases or groups of cases conforming to a particular pattern. Hence, it has been recognized by many writers, including Grant and associates (3), Scadding (4), and Livingstone and co-workers (5), that the acute course originally reported was exceptional, the process being more accurately described as "chronic" in the majority of cases.

More recently the term "fibrosing alveolitis" has been introduced by Scadding (6) on the grounds that the disease appears primarily to affect alveolar lining cells and underlying stroma, and has the features of a chronic inflammatory reaction. Similar pathology has been described in children (3, 7-12), and a familial incidence in several series (12-15) has earned it the term "familial fibrocystic dysplasia" in one paper (12). Other terms used have included "reticular lymphangitis of the lungs" (2) and "diffuse interstitial angiosis" (16). Although it is recognized that the "interstitial" nature of the disease process is fundamental, it is considered that the fibrosis merely represents the end result of a disorder involving other pathologic changes. The term "diffuse interstitial lung disease" is therefore more acceptable.

Recent extensive reviews of the literature (5, 17, 18) make it unnecessary to describe in detail the numerous reports of this disease that have been published in the past decade. These

reports suggest that it is a disease process more common in females, which can occur at any age and which, without treatment, invariably follows a progressive course leading to gradual deterioration and death over a period lasting from a few months to several years.

Since the report by Grant and associates (3), a number of cases have been seen in the three Respiratory Diseases Units in Edinburgh. These are described in this paper.

#### MATERIAL

The series consisted of 42 patients, 22 females and 20 males (ages, 12 to 77 years), who have been seen for the past 12 years. Almost half of the patients presented with symptoms in their seventh decade; and, of the 15 patients under 60 years old, all but 3 were females.

The series was divided into 3 groups: group I, 25 cases in which the diagnosis was histologically proved; group II, 9 cases without histologic proof, but in which the diagnosis was fully supported by the clinical and roentgenologic findings, the results of respiratory function tests, and the subsequent course of the illness; and group III, 8 cases incompletely investigated, in which the diagnosis rested mainly on the roentgenologic changes and the clinical features, and in which there were no symptoms or other findings to suggest an alternative diagnosis.

The diagnosis of diffuse interstitial lung disease was based on five points: (1) history of dyspnea without wheeze or significant episodic variation; (2) finger clubbing; (3) bilateral crepitations on auscultation of the chest, especially when these were described as "superficial" or dry in nature; and (4) respiratory function tests showing a reduction in vital capacity of more than 20 per cent of the predicted value for that patient, and/or arterial hypoxemia with a normal or low  $P_{aCO_2}$  present, either at rest or after exercise.

#### OBSERVATIONS

##### *Pathology*

Lung tissue was obtained for histologic examination from 25 patients: from 12 by lung biopsy at thoracotomy, from 11 at autopsy, and from 2 by lung biopsy and at subsequent autopsy. The pathologic changes found were simi-

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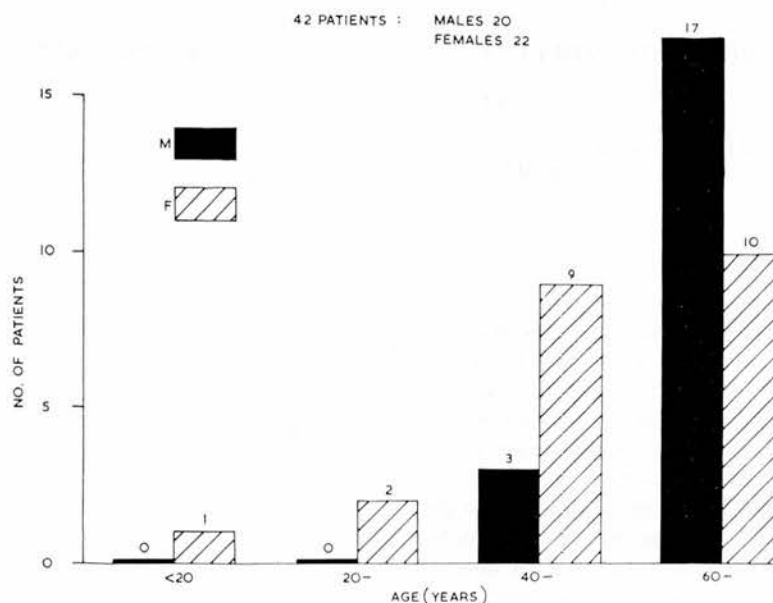


FIG. 1. Age distribution of patients.

lar to those recently described in detailed reports (5, 7, 17). Diffuse fibrosis was the single predominating feature but, in some patients, this was so extensive as to obscure its relationship to alveolar structure. When this was preserved, the fibrosis was interstitial; and, in as many as 18 patients, it was associated with significant infiltration with lymphocytes, plasma cells, and mononuclear cells. Interstitial edema was present in 3 patients. There were changes in the alveolar lining consisting of hyperplasia of the epithelial cells in 6 patients, and of squamous metaplasia in 2. Hyaline membrane formation was not a feature in any patient. In the more densely fibrotic areas, cystic spaces lined by connective tissue had replaced normal alveolar structure.

In 13 patients bronchiolar changes were observed. These consisted mainly of bronchiolar dilatation. In 2 patients marked hyperplasia of bronchiolar epithelium was seen; and, in one patient, there was squamous metaplasia. Vascular changes, with thickening of the vessel wall, chiefly involving the intima, were present in 12 patients.

In the report by Livingstone and associates (5) the pathologic process was divided into five stages. The first three were characterized by complete or partial preservation of normal alveolar architecture, and this was seen in 19

of the patients in this series. In the remaining 6 patients there were varying degrees of disorganization with cyst formation, corresponding to the last two stages.

There is some doubt as to the value of lung biopsy in the assessment of patients with a diffuse bilateral pulmonary abnormality. Open lung biopsy at thoracotomy carries a definite risk in patients with impaired respiratory function, as shown by the occurrence of one post-operative death in this series and another recently reported elsewhere (5). Lung biopsy with the Jack needle is also not without danger (20). Furthermore, with this technique, a very small, and possibly unrepresentative, specimen from one part of lung is obtained. In the vast majority of cases the diagnosis can be made from clinical, roentgenographic, and respiratory function investigations; and, even when the condition is simulated by other diseases, e.g., diffuse pulmonary sarcoidosis, progressive systemic sclerosis, the management is the same. There is, therefore, not a strong case for lung biopsy as a routine diagnostic measure.

It might be expected that the histologic appearances at lung biopsy would be of value in predicting the response of individual patients to corticosteroid therapy. For example, a better response might have been anticipated in those cases with reversible changes such as cellular



infiltration and interstitial edema (7), with preservation of alveolar architecture, than in those where irreversible fibrosis and disorganization had occurred. In the present series, only 2 of the 6 patients in the latter group showed any response at all; but, among 15 patients with apparently early changes, as many as 9 did not improve with treatment. Hence, although lung biopsy can show which patients are unlikely to respond to corticosteroids, the presence of early and apparently reversible changes provides no guarantee that this treatment is likely to succeed.

Some writers have noted the similarity between the changes in the alveolar epithelium in interstitial lung disease and those occurring in the so-called "alveolar cell carcinoma" (pulmonary adenomatosis). Spain (21) quoted 9 cases where this developed on a background of chronic parenchymal fibrosis, predominantly interstitial in type. None of the cases under review here have followed this course, and we know of only one instance (4) in which histologic features of pulmonary adenomatosis were seen in a case otherwise considered typical of chronic diffuse interstitial fibrosis.

On the other hand, bronchial carcinoma has been described occasionally in cases of diffuse interstitial lung disease of the idiopathic type (4, 5, 22) and of the type associated with rheumatoid disease (23, 24). Three of the patients in the present series died from bronchial carcinoma. These were males, aged 61, 62, and 70 years, all of whom had smoked more than 10 cigarettes daily. In 2, the tumor was a peripheral "oat-cell" carcinoma; and, in the third, a squamous carcinoma of a main bronchus was found. The occurrence of 3 cases of bronchial carcinoma among 42 patients with interstitial lung disease, although perhaps noteworthy, is not actually significant because of the age distribution and the small number of cases in the series. It might, however, be profitable to obtain further information on this point by pooling data from a large number of centers.

The possibility of an increased tendency to bronchial carcinoma in idiopathic diffuse interstitial lung disease has also been mentioned by Livingstone and co-workers (5); and, certainly, some of the histologic changes described, notably bronchiolar epithelial hyperplasia and metaplasia, have been considered premalignant by some authorities (25). Reference will be

made later in this paper to the remarkable overgrowth of epithelial cells obtained in one tissue culture from a specimen removed at lung biopsy from one of our patients; a similar occurrence has been reported by Herbert and associates (26). These observations may be relevant to an association between interstitial lung disease and neoplastic change, and would appear to warrant further study.

#### *Clinical Features*

Dyspnea was the dominant presenting symptom in all but 5 of the patients, and only 4 of 42 patients had a normal exercise tolerance. For the purpose of description, dyspnea has been divided into two grades: grade A, slight or moderate dyspnea, which occurred on walking up two flights of stairs at a normal pace but not on walking at a steady pace on the level; and grade B, dyspnea present at rest, or on walking 50 yards or less on the level.

In 29 of the 34 patients whose cases were fully documented, grade B dyspnea was present. Three of the 8 patients with incompletely documented cases had normal exercise tolerance. In these 3 cases the diagnosis was made from routine chest films. With 6 others in the series these patients formed a group of 9 cases with little or no dyspnea. All of these patients were more than 45 years of age and all but 2 were more than 55. Some of them would seem to be examples of a condition affecting older patients, which is later described, in which there are roentgenologic changes consistent with interstitial lung disease without corresponding symptoms or impairment of respiratory function.

Dyspnea in interstitial lung disease is believed to be due to altered mechanics of breathing involving increased work of ventilation. In this series there was no correlation between the degree of dyspnea and the arterial oxygen saturation. A surprising feature is the relatively high vital capacity that may be found in patients with severe dyspnea. In 6 of 30 patients with grade B dyspnea, the vital capacity was, at some stage, over 2 liters. When the lowest vital capacity recorded in each patient was converted into a percentage of the predicted vital capacity, the average value for patients with grade B dyspnea was 59 per cent, although in the less breathless it was 72 per cent. The vital capacity is thus a rough guide to the severity of the disease.

There was little correlation between roentgenographic changes and the degree of dyspnea, although all 19 patients in whom the diaphragm was elevated on maximal inspiration films had grade B dyspnea. Changes in exercise tolerance and respiratory function had to be gross before they were reflected on the chest film, which would seem an insensitive guide to progress.

Cough was a frequent and often distressing complaint, being present in all but 4 patients in this series. It is notable that in patients with interstitial lung disease a bout of irritating coughing often follows deep inspiration. It may be that cough receptors in the lung are sensitive not only to mucosal and pleural stimuli, but also to changes in the mechanism of lung expansion, as in pulmonary venous congestion.

Hemoptysis is described in many reported cases (18, 22, 27). In this series, it was of grave significance, having been recorded in only 3 patients, all of whom had developed bronchial carcinoma.

Over half of the patients in this series suffered from weight loss and/or fatigue. These symptoms are widely reported in the literature. The factors that would seem to be responsible are, first, the increased work of expanding stiffened lungs and, second, chronic hypoxia and its effect on metabolism.

Finger clubbing was present in 35 of the 42 patients, and it apparently preceded the onset of respiratory symptoms by many years in at least 3. Its exact relationship to the pulmonary lesion remains obscure; and, in this study, as in many others, the evidence is confusing. It has been suggested that clubbing is due to failure of the lungs to modify some substance passing through them from the liver and the intestines to the systemic circulation (28). This could be due to some form of arteriovenous shunt in the lung, although there has not been any description of such shunt in interstitial lung disease. Alternatively, it might be associated with the impairment of diffusion of oxygen into the blood. However, the diffusion block theory is now questioned by some authorities (29). In this series clubbing was absent in 4 hypoxic patients, although frequently present in patients with normal oxygen saturation.

Bilateral crepitations were heard on auscultation of the chest in all but 6 patients. In most instances the sounds were described as metallic,

superficial, or dry, with a quality quite distinct from the coarser and "looser" rales heard in resolving pneumonia, bronchitis, and bronchiectasis. The superficiality and fine texture of the sounds suggested that they may have originated more peripherally in the respiratory tract than the latter, and might have been produced by fluid accumulation in the very small air passages, where drainage is hampered by peribronchiolar and interstitial fibrosis.

#### TREATMENT

As in all conditions of unknown etiology, the therapeutic measures hitherto employed in diffuse interstitial lung disease have been either empirical or based on etiologic guesswork. Initially, on the grounds that the disease might be caused by some microorganism, a wide range of antimicrobial agents were tried. Both anti-tuberculosis chemotherapy and corticosteroids came into use during the decade following publication of the report by Hamman and Rich (27), and it is not surprising that they also were employed. Certain writers, including Schechter (30), Read and Holland (31), and Douglas (19), have reported dramatic improvement with corticosteroids in isolated cases; but, in the larger series, such as those of Rubin and Lubliner (17), Scadding (4), and Livingstone and associates (5), only a minority of the patients have benefited from the drugs.

In short, there has been no change from the position in 1956, when Grant and associates (3) concluded that only a small proportion of patients with diffuse interstitial lung disease obtained a useful degree of symptomatic and roentgenographic improvement from corticosteroid therapy! The disease in those patients who did show improvement tended to relapse once the drugs were withdrawn. And, even when no response to treatment occurred, withdrawal was often followed by rapid clinical deterioration.

In order to preserve the last group from unnecessary prolonged corticosteroid therapy, with its attendant side-effects, it would be valuable to have some method of recognizing the patients likely to benefit from corticosteroids. Accordingly, the cases of all of the patients in the present series who received these drugs were reviewed in the hope of obtaining guidance in this matter. Thirty-one of the 42 patients were given corticosteroids. The effect, as judged by

symptomatic, clinical, roentgenographic, and respiratory function assessment, was graded as follows: category 0—no response; category 1—significant improvement without complete remission of symptoms, roentgenographic changes, or impairment of respiratory function (this group included those patients who showed initial improvement but who subsequently deteriorated, such as in the case reported by Nahmias and associates (32)); and category 2—excellent response with complete, or almost complete, remission. The patients were further analyzed as to whether or not their disease had relapsed following withdrawal or reduction of corticosteroids.

The standard regimen currently employed in Edinburgh consists of 20 mg. of prednisolone per day initially, this being reduced by 2.5 or 5 mg. per day after four weeks. Previously a higher initial dose, e.g., 20 to 40 mg. per day, was employed without producing conspicuously better results. Subsequent policy in the individual patient depends upon the response to treatment and on the occurrence of relapse due to withdrawal of the drug.

The results in this series are outlined in table 1. Only 4 patients achieved a category 2 (excellent) response, this group consisting of 3 young women, aged 29, 33 and 41, and one man, aged 52. The disease in the youngest patient only showed a sign of relapse on withdrawal of the drug. Eight patients showed slight or moderate improvement; all but one were female, and only 3 were more than 55 years of age. It was not possible to assess the tendency of the disease to relapse in 2 of these patients, but it was present in 5 of the other 6. Nineteen patients showed no response to corticosteroids. The condition of 5 of these patients deteriorated when the dosage was reduced or the drug was withdrawn. In this group, which consisted of 9

males and 10 females, all except one was more than 55 years old.

These findings confirm previous reports that corticosteroids are more effective in younger patients. However, because of the preponderance of women among our under-60 patients, no conclusion on the bearing of sex on corticosteroid response can be drawn from this series.

Some indication of improvement was usually seen in the first few weeks after treatment was begun. This was especially evident with patients showing a category 2 (excellent) response. In 3 of these patients, subjective and objective improvement was observed within the first ten days. The rate of response was not related to the length of the previous history: it was as rapid in one patient with a four-year history as in another with symptoms of only four months' duration.

The rate of deterioration in one patient, a woman of 61, was almost as rapid. She had suddenly stopped taking her daily dose of 20 mg. of prednisolone, which resulted in a fall in her exercise tolerance, with corresponding roentgenographic deterioration within two weeks. An even more sudden deterioration was reported by Schechter (30) in a 31-year-old male who developed acute respiratory distress and died four days after the withdrawal of corticosteroids.

#### PROGNOSIS AND COURSE

In assessing the prognosis of an individual patient, the first important point is the degree of dyspnea. It has previously been mentioned in this paper that the current series included a group of 9 patients who, when first seen, were not suffering from undue dyspnea but who were referred for a specialist's opinion because of associated symptoms, e.g., chronic cough, or because of the incidental finding of finger clubbing and/or a diffuse roentgenographic abnormality. One of these patients died of an "oat-cell" carcinoma; the other 8 are still living. Three of these, all women less than 50 years old, have since started corticosteroid therapy because of subsequent deterioration. The remaining 5 patients are of particular interest inasmuch as they have survived for over eight years without treatment, and at least 2 have a normal exercise tolerance. These patients have led to the speculation whether there may not

TABLE 1

RESPONSE TO CORTICOSTEROIDS IN IDIOPATHIC  
DIFFUSE INTERSTITIAL LUNG DISEASE

Category of Response	Relapsing	Not Relapsing	Not Assessed	Total
2	1	3	—	4
1	5	1	2	8
0	5	4	10	19
Total	11	8	12	31



TABLE 2  
FATE OF PATIENTS WITH GRADE B DYSPNEA TREATED WITH CORTICOSTEROIDS

Response to Corticosteroids	Category 2	Category 1	Category 0
Total number of cases treated	4	8	16
Average duration of dyspnea before treatment	1 year, 11 months	2 years	2 years, 4 months
Range of duration of dyspnea before treatment	4 months to 4 years	6 months to 9 years	1 month to 10 years
Number of patients alive	4	3	5
Survival time after treatment started	2½, 3½, 6, and 7 years	<6 months, 4 years, 6 years	10 months, 1 year, 1 year, 2 years, 2½ years
Number of deaths among treated group	0	5	11
Deaths due to interstitial lung disease	0	3	6
Time between start of treatment and death	—	2, 5, and 7½ years	<6 months (4 cases), 6 months, 2 years
Death due to causes other than interstitial lung disease	—	2	5

be a benign condition roentgenographically similar to, but clinically distinct from, other forms of interstitial lung disease. Unfortunately, there was no histologic information on these cases, as lung biopsy was not justified on diagnostic or therapeutic grounds.

The fate of the patient with grade B (severe) dyspnea depends upon the response to corticosteroids, as shown in table 2. All those with category 2 (excellent) response were virtually free from symptoms between two and a half and seven years after treatment was started. However, of the 8 patients with a category 1 response, 5 have died. In the 3 patients in whom death was directly due to interstitial lung disease, it occurred two or more years after treatment was started. By contrast, among the 16 patients with grade B dyspnea who showed no response to corticosteroids, 5 deaths directly attributable to interstitial lung disease occurred within a year of starting treatment. In this group, none has yet survived for more than three years. These results could be ascribed to the introduction of corticosteroids at different stages of the disease process; but there was, in fact, no appreciable difference among the three groups with regard to the duration of dyspnea before corticosteroids were employed.

Respiratory insufficiency, either alone or in association with a respiratory infection and/or congestive cardiac failure, accounted for 9 of the 20 deaths. Such an end is an unpleasant one, as the patient literally chokes to death and

(unless heavily sedated) is fully conscious to the last, without the endogenous anesthetic of retained carbon dioxide that eases the terminal bronchitic out of the world.

Sudden unexplained death occurred in 3 patients. In 2 of these patients coronary artery disease was present and might have been responsible, although there was no definite pathologic evidence of recent myocardial infarction. Three other patients died from proved myocardial infarction and 3, from bronchial carcinoma. One 68-year-old man died following lung biopsy, and another death was due to perforation of a duodenal ulcer in a patient receiving corticosteroids.

#### ETIOLOGY

##### *Viral Theory*

In their original description, Hamman and Rich (27) discussed whether their 4 cases of acute diffuse interstitial lung disease could have been caused by a virus. Even now, 20 years later, the evidence for this remains tenuous and circumstantial, being based on four points: failure to find any other cause, such as bacterial infection; nature of the inflammatory reaction; similarity between the histologic picture and that of certain human pneumonias of viral or rickettsial origin; and similarity between the histologic features and those of certain chronic interstitial pneumonias of sheep, which are known to be transmitted by viruses and/or pleuropneumonia-like organisms.



To show that a virus is responsible for the lung changes would require three steps: demonstration that the virus is present in the diseased organ; isolation and identification of this virus; and proof that this virus causes the pathologic changes.

An indirect approach aims at the detection of a rise in serum titer of antibody to virus during the course of the disease. This presupposes that the virus is already known and can be isolated and that the disease process is active during the period of investigation. Neither of these postulates was, however, necessarily acceptable in the cases studied in the present series.

A more direct approach involves examination of specimens obtained at lung biopsy; this was undertaken in 4 patients in the series. Cultures of lung tissue were prepared from three specimens and examined for changes that might indicate the presence of virus. Giant cells (33) were found in all of these, but this does not constitute specific evidence of virus infection (34). A feature of the tissue culture from one specimen (No. 25) was an overgrowth of epithelial cells. This has previously been reported by Herbert and his colleagues (26), and has been discussed elsewhere. Cytopathic changes suggestive of viral infection were not seen in the three original tissue cultures. The fluids obtained from these cultures and those produced by homogenization of lung tissue from all 4 patients were inoculated into tissue cultures of monkey kidney, human epithelium (H.Ep.2), and human embryo lung, and incubated for ten days. Fluids harvested from these cultures were then inoculated into fresh cultures of the same cell lines and incubated for a further period of ten days. All of the cultures so treated were examined for cytopathic changes, but none were found. This confirms the finding of Herbert and associates (26).

Finally, an attempt was made to demonstrate interference activity in the fluids harvested from all of the tissue cultures, using the hemagglutinating property of Echo 11 virus in monkey kidney cells as an indicator. This, again, failed to produce any evidence of the presence of virus.

All of the patients investigated had developed respiratory symptoms at least one year previously. It is still possible that the initial dis-

ease process was due to the presence of virus in lung tissue but that this disappeared at a relatively early stage. Further investigation of early acute cases might help to clarify this point. Such investigations should include techniques designed to detect the presence of pleuropneumonia-like organisms that do not always produce a cytopathic effect in tissue cultures.

#### *Immune Mechanism Disorder*

At a time when a variety of diseases of hitherto unknown pathogenesis have been shown to be associated with disorders of the immune mechanism, it is not surprising that attempts have been made to incriminate these in the pathogenesis of diffuse interstitial lung disease. The evidence for this hypothesis in the present series of cases is briefly summarized below, taking, as a guide, some of the properties of the immune disorders outlined by Mackay and Burnet (35).

*Presence of demonstrable antibody against body components:* The sera of 20 of the patients in this series were examined for evidence of circulating antibodies to thyroid and to gastric mucosa, and for antinuclear factor and nonspecific complement-fixing antibody, by methods previously described (36, 37). Although antibodies were found in the sputum of 5 patients (in 2 patients, to thyroid and in 3, to gastric mucosa), this is not in excess of what might have been expected in a series of normal patients of the same age and sex. Antinuclear factor and rheumatoid factor were not found in any of the cases under review, although both were present in 4 of 6 patients reported elsewhere (38) in whom diffuse interstitial lung disease was associated with rheumatoid arthritis. These findings do not exclude the possibility that autoimmunity played some part in the early stages of the disease, as in most of the cases investigated the lung changes had been present for a considerable time and the interstitial fibrosis may merely have represented the burnt-out relic of a previously active process. In no case was serum obtained during the first month or two of relatively acute progressive interstitial infiltration with cells and fluid. Furthermore, 10 of the patients were receiving corticosteroid therapy at the time the serum was examined, and these agents are known to suppress antibody production.

Sera

The same sera were also tested for evidence of antibody to lung tissue, using the indirect fluorescent antibody technique. The serum under investigation was added to frozen sections taken from a normal portion of lung removed at thoracotomy for bronchial carcinoma. After washing in buffer solutions, the specimens were treated with antihuman gamma globulin conjugated with fluorescein. The sections were then examined under ultraviolet light for localization of dye to specific cells. Such an occurrence would have suggested an immune reaction at that site, but in none of the sera so far tested was significant staining of parenchymal cells observed.

*Co-existence of other conditions believed to be immune disorders:* There have been numerous reports of identical clinical and pathologic manifestations of interstitial lung disease occurring in the connective tissue disorders since the original paper describing this in rheumatoid arthritis (39). The present writers have presented in a separate paper an account of 8 patients with interstitial lung disease and rheumatoid arthritis seen in Edinburgh during the same period as the series now under review (38).

*Infiltration of affected tissue by mononuclear cells, lymphocytes, and plasma cells:* This has been described in 18 of the 25 patients with histologic data. The presence of lymphocytes may be of special significance, as it is thought that these cells may carry cell-bound antibody into the tissues. This is a possible explanation of the failure to demonstrate circulating antibodies in these patients.

*Elevation of serum gamma globulin levels:* It is known that the gamma globulin fraction of the serum proteins contains the majority of circulating antibodies. Elevation of this fraction has been reported in different forms of interstitial lung disease. In the 17 patients in this series in whom the serum electrophoretic pattern was recorded, it was found to be normal (<1.5 gm. per 100 ml.), in 8, slightly raised (1.5 to 2 gm. per 100 ml.) in 3, and considerably raised (>2.0 gm. per 100 ml.) in 6.

*Response to corticosteroid drugs:* Of the 31 patients treated with corticosteroids, only 12 showed a significant response. Here again, a possible explanation is that the deleterious immune process may be active only during the initial stages. This may account for the dra-

matic success of corticosteroids in the one acute case in which they were employed four months after the onset of symptoms.

At present, the case for diffuse interstitial lung disease's being an immunity disorder remains unproved. Although some of these findings are suggestive, it is difficult to be certain whether the pathologic and serologic changes are of pathogenetic significance or only the result of some form of tissue damage.

It may, indeed, be that the condition merely represents the nonspecific response of a mature tissue that can react in only a few ways to a variety of different stimuli.

#### SUMMARY

Forty-two cases of idiopathic interstitial lung disease are described. The patients consisted of 22 females and 20 males between the ages of 12 and 77 years.

In 25 of these patients, the diagnosis was confirmed by histologic examination of lung tissue removed at thoracotomy or at autopsy. The principal pathologic changes were diffuse interstitial fibrosis and infiltration with lymphocytes, plasma cells, and mononuclear cells. Lung biopsy appeared to be of limited value, both as a diagnostic procedure and in predicting the response to corticosteroid therapy.

The main symptoms were dyspnea, cough, weight loss, and fatigue. The principal physical signs consisted of dry, "superficial" crepitations on auscultation of the chest and finger clubbing.

Thirty-one of the 42 patients received corticosteroid therapy. Four obtained complete remission of symptoms, and 8, slight to moderate improvement. Relapse, or further deterioration of the disease, followed reduction of the dosage or withdrawal of the corticosteroids in 6 patients who responded to treatment and in 5 who did not respond.

The prognosis seemed to depend upon the degree of dyspnea and, in those patients with significant dyspnea, upon the response to the corticosteroids. Five patients with little or no dyspnea have survived for more than eight years without treatment.

No evidence of viral infection was found in lung tissue obtained at biopsy, and no circulating antilung antibodies were identified in the

sera. It is postulated that the condition may represent the end result of a pathologic reaction to a number of different stimuli that are no longer recognizable by the time the pulmonary changes are fully established.

#### SUMARIO

##### *Enfermedad Difusa Intersticial Idiopática: Informe de Cuarenta y Dos Casos*

Se describen cuarenta y dos casos de enfermedad difusa intersticial idiopática. Los pacientes consistían de 22 hembras y 20 varones, entre las edades de 12 a 77 años.

En 25 de ellos, se confirmó el diagnóstico por el estudio histológico de tejido pulmonar obtenido por la toracotomía o la autopsia. Los cambios histopatológicos predominantes fueron fibrosis intersticial difusa e infiltración linfocítica y de células plasmáticas y mononucleares. La biopsia pulmonar fué aparentemente de utilidad limitada, tanto en el diagnóstico como en la predicción de la respuesta a la terapia por corticoesteroides.

Los síntomas predominantes fueron disnea, tos, pérdida de peso, cansancio. Los hallazgos principales fueron estertores crepitantes y dedos hipocráticos.

Treinta y un pacientes recibieron esteroides; en 4 hubo remisión completa y en 8, mejoría de leve a moderada. Se observó recidiva o progreso de la enfermedad en 6 pacientes en que se redujo la dosis o se eliminó la droga y en 5 sin respuesta favorable.

El pronóstico parecía depender de la severidad de la disnea y del efecto a los esteroides. Cinco pacientes con poca o ninguna disnea han sobrevivido más de ocho años sin terapia alguna.

El tejido pulmonar obtenido mediante biopsia no reveló infección viral y no se hallaron anticuerpos anti-pulmón en el suero. Se postula que la enfermedad puede ser la fase final de una reacción patológica provocada por diversos estímulos que dejan de ser reconocibles una vez manifestada la neumopatía.

#### RESUME

##### *Maladie interstitielle diffuse idiopathique du poumon: relation de quarante deux cas*

Quarante-deux cas de maladie interstitielle diffuse idiopathique du poumon sont relatés. Il s'agit de 22 malades de sexe féminin et de 20 malades de sexe masculin, âgés de 12 à 17 ans.

Chez 25 de ces malades, le diagnostic a été confirmé par l'examen histologique du tissu pulmonaire prélevé lors de la thoracotomie ou de l'autop-

sie. Les modifications pathologiques principales qui ont été notées sont la fibrose interstitielle diffuse et l'infiltration à lymphocytes, plasmocytes et cellules mononucléaires. La biopsie du poumon est apparue comme dotée d'une portée limitée, tant en ce qui concerne sa valeur comme procédé de diagnostic que pour les possibilités qu'elle pourrait offrir pour prédire la réponse à un traitement par les corticostéroïdes.

Les symptômes majeurs ont été la dyspnée, la toux, la perte de poids et la fatigue. Les signes physiques principaux ont consisté en crépitations sèches "superficielles" à l'auscultation et à la percussion digitale du thorax.

Trente et un des 42 malades étaient soumis à la thérapeutique par les corticostéroïdes. Quatre malades en ont obtenu une disparition complète des symptômes et 8, une amélioration pouvant être qualifiée de légère à modérée. Chez 6 malades qui répondaient au traitement et chez 5 qui ne réagissaient pas, une réduction de la posologie ou la suppression des corticostéroïdes a été suivie par une récurrence ou par une aggravation de la maladie.

Le pronostic a semblé dépendre du degré de dyspnée et aussi, chez les malades avec dyspnée prononcée, de la réponse aux corticostéroïdes. Cinq malades qui ne présentaient pas ou que peu de dyspnée ont survécu sans traitement pendant plus de huit ans.

On n'a pas pu mettre en évidence une infection virale dans les tissus pulmonaires prélevés par biopsie. On n'a pas identifié d'anticorps anti-poumon circulant dans le sérum. On considère que ce syndrome peut représenter le résultat terminal d'une réaction pathologique à quantité d'excitations différentes qu'il n'est plus possible d'en reconnaître une fois que les modifications pulmonaires sont complètement établies.

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CHAPTER VII

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THE POSSIBLE ASSOCIATION BETWEEN AUTOIMMUNITY  
AND DISORDERS OF THE SEX CHROMOSOMES

"Autoimmunity in women with sex chromosome  
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## AUTOIMMUNITY IN WOMEN WITH SEX CHROMOSOME ANEUPLOIDY AND IN THEIR PARENTS COMPARED TO CONTROLS

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### SUMMARY

The incidence of thyroid and gastric antibodies in patients with clinical and cytogenetic features of gonadal dysgenesis, in doubly chromatin positive women and in the parents of patients with gonadal dysgenesis was not found to be significantly higher than in controls matched for age.

The incidence of antibodies appeared to be appreciably higher in mosaics and in mosaics whose sex chromosome complement included an isochromosome of the long arm of the X. Failure to establish the significance of these raised incidences may be due to inadequate numbers.

The discrepancy between the findings in this series and in previously reported series is discussed.

### INTRODUCTION

In 1961, Engel & Forbes reported a patient with the clinical features of Turner's syndrome with an XX sex chromosome complement, one X being an isochromosome (46,XXqi), who had previously suffered from and been treated for Hashimoto's disease of the thyroid. Two similar cases were reported by Sparkes & Motulsky (1963) and another by Grumbach & Morishima (1964). In the same year Williams, Engel & Forbes (1964) reported a series of twenty-five patients who had ovarian dysgenesis and a variety of sex chromosome abnormalities of whom three had evidence of chronic thyroiditis and thirteen had positive tests for circulating thyroid antibodies. Thyroid antibodies were also frequently found in close relatives of these patients by Fialkow (1966) and more recently by Vallotton & Forbes (1967) who extended the studies on the series first described by Engel & Forbes (1965). Doniach, Roitt & Polani (1968) have also reported that patients with ovarian dysgenesis and abnormal sex chromosomes may be particularly prone to develop thyroid antibodies and also concluded that different chromosomal subgroups differ in their propensities to antibody production. The parents of the patients did not, however, appear to have a raised incidence of antibodies in relation to their ages. In this paper we report on the findings of

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thyroid, gastric and other organ-specific and non-organ-specific antibodies among patients with gonadal dysgenesis and among women with a 47,XXX sex chromosome complement. When the parents of patients with gonadal dysgenesis were alive and accessible for investigation, these too were studied. The frequency of thyroid and gastric antibodies in these subjects has been compared with the results obtained by the same laboratory among healthy blood donors of the same age and sex.

#### PATIENTS STUDIED

The patients had all been referred to the Registry of Abnormal Karyotypes at the Clinical and Population Cytogenetics Research Unit in Edinburgh. Their age distribution in decades is given in Table 1. Fifty-one of the patients had gonadal dysgenesis (Turner's syndrome) and fourteen were doubly chromatin positive on examination of buccal smears.

##### *Gonadal dysgenesis*

This diagnosis was based on a history of amenorrhoea, failure to develop secondary sexual characteristics at puberty, short stature and an abnormal sex chromosome complement compatible with this diagnosis. Of the fifty-one patients, thirty-nine had been identified when they presented with primary amenorrhoea, eight had been referred for investigation of failure to grow, two for investigation of suspected thyroid disturbances, one for secondary amenorrhoea and one for obesity.

Twenty-one of these patients had a 45,X karyotype and twenty-four had a chromosome mosaicism in which one cell line had a 45,X karyotype. The karyotypes of these patients is shown in full in Table 1. The structural abnormalities and the count distributions are described elsewhere under the patients' case numbers in the Registry of Abnormal Karyotypes (Court Brown *et al.*, 1964; and to be published) given in a footnote to Table 1.

None of the fifty-one patients had clinical evidence of thyroid disease at the time of this investigation. However, one had been diagnosed as suffering from thyrotoxicosis (PBI = 13.4  $\mu\text{g}/100\text{ ml}$ ) when she was originally referred for chromosome studies, since when she has been successfully treated with anti-thyroid drugs for 18 months. Eight of the fifty-one patients had been suspected of suffering from hypothyroidism including seven who had been treated with thyroid extract but in one instance only was the diagnosis confirmed on re-investigation and was disproved in the remainder. Another patient had a thyroglossal cyst excised in childhood. In two patients (108/61; 34/63) the levels of protein bound iodine (9.2 and 11.2  $\mu\text{g}/100\text{ ml}$ ) were above the upper limit of normal set by the laboratory. The  $^{131}\text{I}$  uptakes at 6 hr were 52 and 31%, respectively. Neither patient had clinical evidence of thyroid disease and in both patients the  $\text{PB}^{131}\text{I}$  levels were less than 0.4% of administered dose at 48 hr. In the remaining thirty-one patients whose PBI was estimated, the values (4.0–8.2  $\mu\text{g}$ ; mean 6.4  $\mu\text{g}/100\text{ ml}$ ) were within the normal limits.

##### *Doubly chromatin-positive females*

Fourteen women included in the study had been identified as doubly chromatin positive during the routine examination of buccal mucosal smears obtained from patients attending general hospital clinics and from patients at mental subnormality hospitals. In eleven of these, the karyotype was 47,XXX and three were mosaics (Table 1). Seven patients were



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mentally retarded and one of these patients had a large firm diffuse goitre and had been given thyroid replacement therapy for several years. Among the remaining patients one is

TABLE 1. Chromosome constitution and ages of fifty-one females with evidence of ovarian dysgenesis and fourteen females who were doubly chromatin positive

Chromosome karyotype	Age (years)						Total
	20	20-29	30-39	40-49	50-59	59	
Gonadal dysgenesis							
45,X	3	12	6	0	0	0	21
46,XXqi	0	5	0	0	0	0	5
46,XXp-	1	0	0	0	0	0	1
Mosaics							
45,X/46,XX	0	1	1	0	0	0	2
45,X/46,XX/47,XXX	0	0	0	0	0	1	1
45,X/46,XY	1	3	0	0	0	0	4
45,X/47,XY	0	0	0	1	0	0	1
45,X/46,XXqi	2	6	1	1	0	0	10
45,X/46,XXqi/47,XXqiXqi	1	0	0	0	0	0	1
45,X/46,XXr	1	1	0	0	0	0	2
45,X/46,XXmar	0	2	0	0	0	0	2
45,X/46,Xr+	0	0	0	1	0	0	1
Doubly chromatin positive females							
47,XXX	1	3	2	2	1	2	11
45,X/47,XXX	0	0	1	0	1	0	2
46,XX/47,XXX	0	0	0	0	1	0	1
Case No. in registry of abnormal karyotypes							
45,X	9/59, 16/59, 54/60, 55/60, 111/60, 162/60, 169/60, 108/61, 169/61, 2/62, 40/62, 95/62, 3/63, 116/63, 61/64, 97/64, 152/64, 78/65, 137/65, 145/65, 314/67.						
46,XXqi	35/60, 5/63, 34/63, 97/63, 90/64.						
46,XXp-	230/66.						
45,X/46,XX	74/61, 154/64.						
45,X/46,XX/47,XXX	87/66.						
45,X/46,XY	65/62, 59/64, 179/64, 118/65.						
45,X/47,XY	152/60.						
45,X/46,XXqi	2/59, 41/60, 168/61, 179/61, 100/62, 101/63, 10/64, 143/65, 188/66, 240/67.						
45,X/46,XXqi/47,XXqiXqi	62/63.						
45,X/46,XXr	98/65, 140/65.						
45,X/46,XXmar	95/64, 42/60.						
45,X/46,Xr+	229/66.						
47,XXX	55/63, 177/64, 54/66, 52/66, 128/64, 124/64, 51/65, 7/65, 5/65, 259/67, 210/67.						
45,X/47,XXX	6/65, 99/66.						
46,XX/47,XXX	180/64.						

said to have suffered from thyrotoxicosis (confirmatory data not available) and is at present being treated with antithyroid drugs.

*Parents of patients with gonadal dysgenesis*

Twenty-seven mothers (age range 38–69, mean age 53) and twenty-four fathers (age range 40–77, mean age 55) of patients with gonadal dysgenesis were studied. One of the mothers had suffered from thyrotoxicosis and been treated by partial thyroidectomy and two had diffuse thyroid enlargement. None of the fathers examined had clinical evidence of thyroid disease.

TABLE 2. Incidence of thyroid and gastric antibodies in healthy controls

Age	No. of subjects	Thyroid antibodies						Gastric parietal cell antibodies		Total	
		Thyroglobulin*		Cytoplasmic†		Total					
		No.	%	No.	%	No.	%	No.	%	No.	%
Females											
10-19	53	1	1.9	1	1.9	2	3.8	2	3.8	3	5.7
20-29	87	7	8.0	3	3.4	9	10.3	1	1.1	8	9.2
30-39	78	5	6.4	8	10.0	9	11.5	7	8.9	15	19.2
40-49	70	6	8.0	11	15.7	14	20.0	6	8.0	17	24.3
50-59	70	12	17.0	16	22.8	10	28.5	7	10.0	22	30.1
60-69	46	7	15.2	7	15.2	11	23.9	6	13.0	14	30.4
Total	404	38	9.4	46	11.4	65	16.1	29	7.2	79	19.6
Males											
10-19	47	Nil	Nil	2	4.3	2	4.3	2	4.3	3	6.4
20-29	75	3	4.0	Nil	Nil	3	4.0	2	2.7	4	5.3
30-39	74	2	2.7	Nil	Nil	2	2.7	Nil	Nil	2	2.7
40-49	84	2	2.4	6	7.1	7	8.3	8	9.5	14	16.7
50-59	58	1	1.7	3	5.2	3	5.2	Nil	Nil	3	5.2
60-69	38	1	2.6	Nil	Nil	1	2.6	1	2.6	2	5.3
Total	376	9	2.4	11	2.9	18	4.8	13	3.5	28	7.4

\* TCH titre  $\geq 1:25$ .

† Positive immunofluorescent staining  $\pm$  positive complement fixation test.

*Controls*

The control series consisted of 404 women and 376 men aged between 10 and 69 years. Those between the ages of 20–59 were attending blood transfusion donor sessions. Those at the extremes of the age range were hospital control subjects who had no clinical evidence of thyroid disease, pernicious anaemia, adrenal insufficiency, hypoparathyroidism, diabetes mellitus or any history suggestive of a gross defect of gonadal function and who had no gross growth defect. The age distribution by decades and the numbers with antibodies is given in Table 2.

## METHODS

The sera of the patients were examined for antibodies to thyroglobulin by the tanned cell haemagglutination test (TCH) (Fulthorpe *et al.*, 1961) with serum dilutions of 1:5, 1:25 and serial ten-fold dilutions thereafter. Titres  $\geq 1:25$  were regarded as positive. Antibody to thyroid cytoplasmic antigen and antibody to the cytoplasm of gastric parietal

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cells were examined by the indirect immunofluorescence test using unfixed sections of human thyrotoxic thyroid tissue and gastric mucosa from the body of human stomach using undiluted serum and anti IgG conjugated with fluorescein isothiocyanate (Irvine, 1963; Irvine, Stewart & Scarth, 1967). The method of preparation and properties of the conjugate are described elsewhere (Irvine, Chan & Williams, 1969). Sections of rat kidney were included in each test as a control for specificity and, in particular, for the detection of mitochondrial antibodies (Doniach *et al.*, 1966) and as a further check for antinuclear factors. Tests giving positive immunofluorescence with thyroid or gastric sections were tested and titrated by the method of complement fixation using doubling dilutions of serum starting at 1:2, Takatsy plates, 2 MHD of complement and 50% haemolysis as end point (Irvine, 1966). The immunofluorescence sections were also checked for the presence or absence of nuclear staining.

The sera of forty-nine of the patients with gonadal dysgenesis and of the fourteen women who were doubly chromatin positive were also tested against unfixed cryostat sections of human and rabbit ovary in the follicular and in the luteal phase, human and rabbit testis, human placenta and human adrenal (Irvine *et al.*, 1968, 1969).

## RESULTS

### *Gonadal dysgenesis*

#### *Thyroid antibodies*

Of the fifty-one patients with clinical and cytogenetic features of gonadal dysgenesis, eight had antibodies to at least one thyroid constituent. This is an incidence of 15.7% as compared with an incidence of 17.0% among 305 controls. Forty-seven of the patients were under the age of 40 years and of these seven had thyroid antibodies. This is an incidence of 14.9% which is higher than the incidence of 10.9% in control women aged 20-39 years but the difference is not significant.

Two of the twenty-one patients with a 45,X karyotype (9.6%) had thyroid antibodies which is not significantly different from controls in the same age group.

Of the twenty-four patients of all ages with mosaicism, six (25%) had thyroid antibodies and, of the twenty who were under 40 years, five (25%) had thyroid antibodies. Both incidences are higher than among female controls (16.1 and 9.2%, respectively) but the differences are not significant. All six patients with thyroid antibodies had a 45,X cell line and three of them also had a 46,XXqi cell line. The three thyroid antibody patients with a 46,XXqi cell line were found among a total of eleven patients who were 45,X/46,XXqi mosaics, an incidence of 27.1%. The other three mosaics who were positive for thyroid antibodies were constituted as follows: 45,X/46,XX; 45,X/46,XY and 45,X/46,X+mar, the second cell line being different in each case. The finding of three patients with thyroid antibodies out of eleven with a 45,X/46,XXqi constitution (27.1%) was not significantly different than the findings among the controls. Finally, no patient was found to have thyroid antibodies out of the five studied whose sex chromosome complement was 46,XXqi without mosaicism.

The tanned red cell haemagglutination titre did not exceed 250 in any of the patients with antibodies. This was also the highest titre among controls under the age of 40 but there were three control women over this age who had titres greater than 250. The highest complement fixation titre of cytoplasmic antibodies among all the patients and among controls under 40 years was 32, but seven of the 305 control subjects over the age of 40 years had titres greater than 32.

*Gastric antibodies*

Three of the fifty-one patients with gonadal dysgenesis (5.9%) had gastric parietal cell antibodies as compared with 7.2% of the female controls. The three patients were between 20–33 years of age so that the incidence is 6.4% as compared with 4.8% of the female controls aged 20–39 years. This difference is not significant. The karyotypes of the patients with gastric parietal cell antibodies is shown in Table 3 but the numbers are too small to consider further analysis. The complement fixation titres on the three patients did not exceed 1:32.

The sera of forty-nine of the fifty-one patients were tested for intrinsic factor antibody I and all found to be negative.

*Ovarian, testicular, placental and adrenal antibodies*

The sera of forty-nine of the patients with gonadal dysgenesis gave negative results for antibodies to ova, theca interna, interstitial cells and corpus luteum of ovary and interstitial cells and spermatids of testis, as well as negative results with placental and adrenal tissue. The sera from the remaining two patients were not available for testing for these antibodies.

*Mitochondrial antibody and antinuclear factors*

The sera of forty-nine of the patients with gonadal dysgenesis were negative for mitochondrial antibody and for antinuclear factors. The sera of the remaining two patients were not available for this purpose.

*Doubly chromatin positive females*

Two of the fourteen patients who were doubly chromatin positive had thyroid antibodies and one of these had gastric antibodies. Both patients were over the age of 40 and one was identified when attending a hospital for treatment of hyperthyroidism. The other patient, a 45,X/47,XXX mosaic, had clinical thyroiditis and a TRC titre of 2500. This incidence of antibodies among doubly chromatin females is not significantly raised. No antibodies to ovarian, testicular, placental or adrenal tissue were detected in these patients.

The results of thyroid and gastric antibody tests are detailed in Table 3 and summarized in Table 4.

*Parents of patients with gonadal dysgenesis**Thyroid antibodies*

Of the twenty-seven mothers examined, five had circulating thyroid antibodies, an incidence of 18.5% which compares with an incidence of 18.7% in control women aged 40–69 years. Two of these with antibodies were the mothers of patients with a 45,X karyotype and three were the mothers of patients whose karyotype included a 46,XXqi cell line. One of the daughters with a 45,X karyotype also had thyroid antibodies.

Of the twenty-four fathers examined, one had thyroid antibodies. His daughter who was a mosaic with a 46,XXqi cell line had no antibodies and neither had her mother. An incidence of one in twenty-four males (4.2%) of this age is lower than in the male control subjects aged 40–69 (6.1%) or in the population study reported by Dingle *et al.* (1966).



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TABLE 3. Subjects with positive thyroid and gastric antibody tests

Type	Registry No.	Age	Chromosome complement	Thyroid antibodies				Gastric parietal cell antibody	
				Thyroglobulin (tanned red cell titre)	Epithelial cytoplasm		Complement fixation titre	Immunofluorescence	Complement fixation titre
					Immunofluorescence	Complement fixation titre			
Group I	16/59	28	45,X	5	+	2	+	+	2
	2/62	23	45,X	25	+	4	Neg	Neg	Neg
	61/64	20	45,X	Neg	Neg	Neg	Neg	Neg	32
Mosaics	74/61	24	45,X/46,XX	250	+	8	Neg	Neg	Neg
	59/64	22	45,X/46,XY	25	+	32	Neg	Neg	Neg
	2/59	41	45,X/46,XXqi	5	+	2	Neg	Neg	Neg
	100/62	20	45,X/46,XXqi	250	Neg	Neg	Neg	Neg	Neg
	62/63	18	45,X/46,XXqi/47,XXqiXXqi	25	+	Neg	Neg	Neg	Neg
	95/64	20	45,X/46,XXmar	20	+	Neg	Neg	Neg	Neg
	143/65	33	45,X/46,XXqi	Neg	Neg	Neg	+	+	16
Doubly chromatin positive females	55/63	46	47,XXX	Neg	+	4	+	+	16
	6/65	51	45,X/47,XXX	2500	+	64	Neg	Neg	Neg

*Gastric antibodies*

Three of the twenty-seven mothers (11.1%) and none of the fathers had gastric parietal cell antibodies. These findings do not differ from controls in the same age range, 10.2% of the control women and 5% of the control males age 40-69 years having gastric antibodies.

## DISCUSSION

In a group of fifty-one patients with clinical and cytogenetic features of gonadal dysgenesis (Turner's syndrome) and in fourteen women whose buccal smears were doubly chromatin positive, the incidence of thyroid and gastric antibodies was not significantly raised above that in normal controls. Antibodies were also not detected more frequently in a group of parents of patients with gonadal dysgenesis. These findings are in striking contrast to those of Vallotton & Forbes (1967) and of Doniach *et al.* (1968) who found the overall incidence of thyroid antibodies amongst patients with gonadal dysgenesis to be raised (51 and 39.7%, respectively).

TABLE 4. Incidence of thyroid and gastric antibodies with different sex chromosome constitution

Karyotype	No. of patients	Thyroid antibodies		Gastric antibodies	
		No.	%	No.	%
45,X	21	2	9.6	2	9.6
46,XXqi	5	0	0	0	0
46,XXp-	1	0	0	0	0
All mosaics	24	6	2.5	1	4.2
Mosaics with XXqi cell line	11	3	27.1	1	9
Doubly chromatin positive females	14	2	18.3	1	14.2
Female controls	404	65	16.1	29	7.2

The patients we studied were not randomly selected samples of patients with Turner's syndrome or of doubly chromatin positive women but we are not aware of any factors in the selection of the patients which would exclude those with thyroid antibodies. Our patients were considerably younger than those of Vallotton & Forbes, the respective mean ages being 26 and 42 years. This could be an important difference as it is well known that the incidence of antibodies increases with age, but when this younger age of our patients is allowed for and the results compared with findings in women under the age of 40, it was still not possible to demonstrate any increase in the incidence of antibodies. Furthermore the patients studied by Doniach *et al.* (1968) were in fact younger than our patients so the discrepancy with their series cannot be attributed to an age difference.

Our patients could be equally divided into those who had presented at endocrine clinics and those who had presented at gynaecology clinics and the incidence of antibodies was the same in patients from both sources. All but four patients were under investigation for primary amenorrhoea or for failure to grow. Many of the patients studied by Vallotton & Forbes (1967), however, were attending hospital for a variety of conditions which may or may not be associated with the chromosomal abnormality (Engel & Forbes, 1965). A higher

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incidence of antibodies has been reported amongst women in the hospital populations by Hackett, Beech & Forbes (1960) so that it is possible that the way in which patients with gonadal dysgenesis present may influence the incidence of thyroid antibodies. Unfortunately the mode of presentation of the patients is not recorded by Doniach *et al.* (1968).

Among our patients only the mosaics appeared to have an appreciably higher incidence of thyroid antibodies. Although this increase was not significant, this could be due to insufficient numbers, and it is noteworthy that Doniach and her colleagues also found the highest incidence of antibodies among patients with sex chromosome mosaicism. The largest subgroup of the mosaics we studied had an XXqi cell line and half the antibody positive cases were in this group, whilst the others were distributed among mosaics with a variety of second cell lines. It is of interest that cases which first drew attention to a possible association between autoimmune thyroiditis and Turner's syndrome were remarkable in that they all had a sex chromosome complement which included an isochromosome of the long arm of the X, and it was the possible association with additional X chromosome material that prompted the inclusion of women with XXX sex chromosomes in this survey. It may be significant that the only patient with clinical thyroiditis was in this group.

One of us (Irvine *et al.*, 1968) has reported the incidence of two cases of gonadal dysgenesis with normal chromosome constitution in whom there was a strong association with autoimmune disease; idiopathic adrenal atrophy, idiopathic hypoparathyroidism in the first case and these conditions plus primary atrophic hypothyroidism and pernicious anaemia in the second. Both of these patients had a high incidence of autoantibodies of the organ-specific type in the serum.

The parents of only twenty-seven patients with gonadal dysgenesis were studied but again we are not aware of any circumstances which would lead to the exclusion of those having thyroid antibodies and we cannot account for the difference between the incidences of antibodies in this series and in the parents studied by Vallotton & Forbes (1967). Doniach *et al.* (1968), however, were also unable to show a raised incidence of antibodies in parents of patients with gonadal dysgenesis.

It is difficult to account for the differences between our findings in patients with gonadal dysgenesis and those previously reported, except on the basis of differences in the selection of patients. It is clear that in order to determine the significance of apparently high incidences of antibodies it is important that comparisons be made with controls matched not only for age but also for mode of presentation. However, the relatively high incidence of antibodies to thyroid and gastric mucosal cells in the general population will probably make it necessary to study large numbers in order to establish or exclude significant differences. The negative findings in tests for other organ-specific antibodies do not suggest, however, that there is an increased risk of autoimmunity in general among these patients.

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CHAPTER VIII

CHAPTER VIIITHE RELATION OF THE THYMUS TO AUTOIMMUNE DISEASE IN MAN

"The Thymus in Thyroid Disease"  
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## THE THYMUS IN THYROID DISEASE

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## THE THYMUS IN THYROID DISEASE

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THE thymus is generally believed to be situated in the mediastinum, and to be inaccessible unless by splitting the sternum. A new approach to the thymus through a cervical incision, developed at the Royal Infirmary, Aberdeen, has allowed us to obtain thymic material for histological study from patients with various thyroid disorders, including autoimmune thyroid disease.

### Methods

*Operative technique.*—A biopsy specimen was taken from the thymus of 103 patients undergoing thyroid surgery through the standard thyroidectomy incision at Aberdeen Royal Infirmary. The technique, which will be described in detail elsewhere (Gunn and Michie 1964), depends upon identification of the cornua of the thymus in the fibrofatty tissue at the root of the neck. It may readily be found lying on the inferior thyroid veins, immediately deep to the lower portion of the sternothyroid muscles. A constant fibrous cord connects the apex of the thymus to the inferior aspect of the thyroid lobe, and it is along this "thyrothymic axis" that the inferior parathyroid is generally found. This parathyroid often surmounts the thymic apex, and risk of interference is greatly reduced by biopsy of the thymic lobe 0.5 cm. below its apex.

Tissues for histology were fixed in 4% formaldehyde-acetate. Sections were stained with hæmalum and eosin, Unna-Pappenheim, and the Gordon and Sweet method for reticulin fibres.

### Results

*Thymus.*—One or two blocks were taken from each biopsy

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specimen. On histological section, thymus tissue was demonstrated in 95 specimens (92%). The remainder revealed only fibrofatty tissue. There was no doubt surgically that thymus had been identified, and this may represent the state of affairs in the apex of the thymus in a proportion of cases. The thymus was considered abnormal when lymphoid follicles with active germinal centres were seen in the medulla. The follicles were usually small, and they sometimes had irregular margins (fig. 1), in contrast to the large regular follicles characteristically found in myasthenia gravis (fig. 2).

Medullary lymphoid follicles were identified in thymic biopsy specimens from 18 patients of whom 16 had thyrotoxicosis, 1 non-toxic nodular goitre, and 1 Hashimoto's disease without evidence of hyperthyroidism:

Thyroid disease	Thymus medullary lymphoid follicles	
	Absent	Present
Thyrotoxicosis ..	34*	16
Non-toxic goitre ..	43	2*
Total .. ..	77	18

\* One patient had Hashimoto's disease.

The only other case of Hashimoto's disease included in the series was thyrotoxic, but the thymus-biopsy material showed no abnormality. The prevalence of medullary lymphoid follicles in the thymus was much greater in thyrotoxic patients than in those with non-toxic goitre ( $P < 0.01$ ).

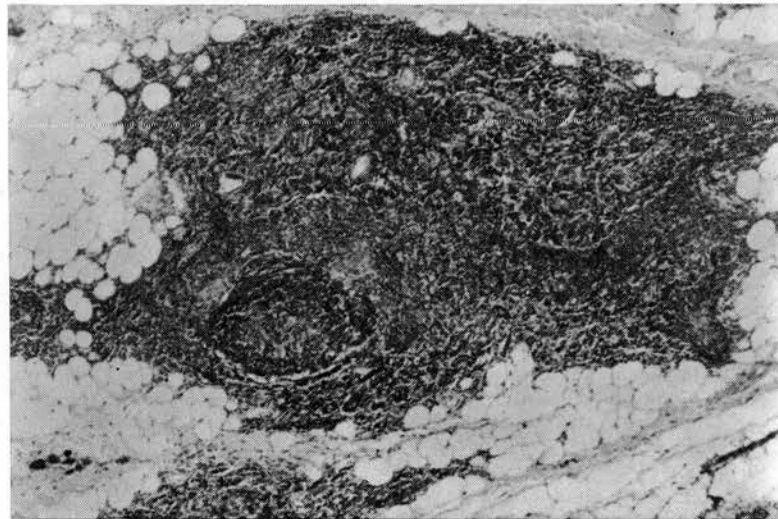


Fig. 1—Thymus from a patient with thyrotoxicosis, showing characteristically small lymphoid follicle in the medulla. (Hæmalum and eosin  $\times 60$ .)

*Thyroid histology.*—An average of four blocks was taken from each specimen of thyroid. Lymphoid follicles with germinal centres were identified in thyroids from 36 patients in the series. The prevalence of thymic medullary lymphoid follicles was greater in these patients than in the remainder of the series ( $p < 0.01$ ):

<i>Thyroid lymphoid follicles</i>			<i>Thymus medullary lymphoid follicles</i>	
Present	..	..	Absent	Present
Present	..	..	23	13
Absent	..	..	54	5
Total	..	..	77	18

It could be argued that this may not be a valid association because the group of patients with thyroid lymphoid follicles will include an unduly high proportion of thyrotoxic patients in whom thymic medullary lymphoid follicles are common. We have therefore investigated the prevalence of thyroid and thymic lymphoid follicles in our patients with thyrotoxicosis, and shown that in this disease there is an association between these abnormalities ( $p < 0.05$ ):

<i>Thyroid lymphoid follicles</i>			<i>Thymus medullary lymphoid follicles</i>	
Present	..	..	Absent	Present
Present	..	..	11	11
Absent	..	..	23	5
Total	..	..	34	16

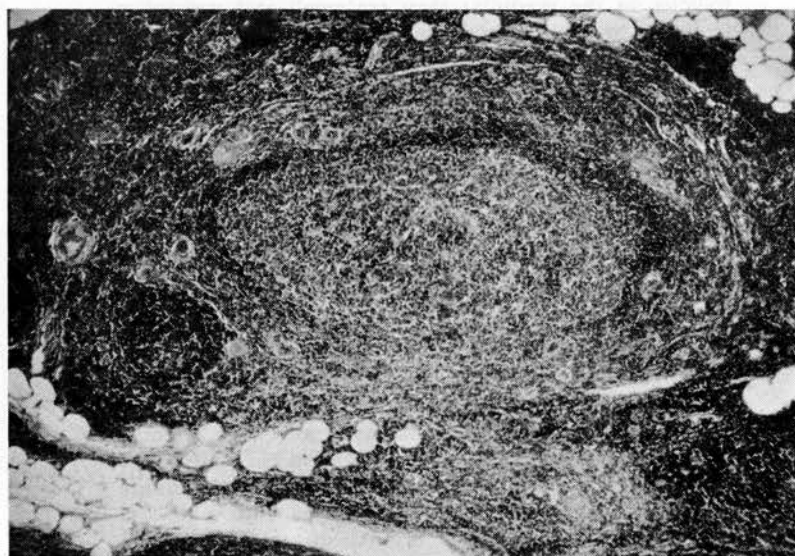


Fig. 2—Thymus from a patient with myasthenia gravis, showing large lymphoid follicle in the medulla. (Hæmalum and eosin  $\times 60$ .)

*Antithyroid antibodies.*—Sera from 93 patients were tested, (a) for anti-thyroglobulin by the tanned-red-cell agglutination technique and the Ouchterlony-precipitin test, (b) for antibody to thyroid microsomes by the complement-fixation technique and immunofluorescence, and (c) for antibody to the second colloid antigen (anti-CA<sub>2</sub>) by immunofluorescence. The apparent correlation in the whole series between thymic medullary lymphoid-follicle formation and the presence of thyroid antibodies ( $P < 0.05$ ) does not hold when the group of thyrotoxic patients is studied ( $P > 0.2$ ), and it may therefore be owing to the higher proportion of thyrotoxic patients in the group with thymic abnormalities:

Antithyroid antibodies	Thymus medullary lymphoid follicles		Thyrototoxicosis	
	Whole series	Thyrototoxicosis	Thyrototoxicosis	Thyrototoxicosis
Present	.. ..	22	11	13
Absent	.. ..	53	7	20
Total	.. ..	75	18	33
				16

#### Discussion

Although there have been previous reports of lymphoid follicles in the thymus in thyrotoxicosis (see Sloan 1943), this is the first in-vivo study of the thymus in a large group of patients with pathological changes in the thyroid. The most prominent thymic abnormality in this series of patients with thyroid disease is the presence of lymphoid follicles in the medulla: they are small and somewhat irregular in outline, and they are formed of a halo of lymphocytes surrounding an active germinal centre in which mitoses are seen infrequently. These follicles are similar to the small medullary lymphoid follicles in the thymus of patients with rheumatoid arthritis (Burnet and Mackay 1962) and systemic lupus erythematosus (Mackay et al. 1963, 1964), and they have the same general structure as the large active germinal centres found in the thymic medulla in myasthenia gravis (Castleman 1960, White and Marshall 1962, Strauss 1963). In view of the important role of the thymus in normal and abnormal immunological reactions (Miller 1961, 1962, 1963, Burnet 1962, Burnet and Holmes 1963), it is noteworthy that thymic medullary lymphoid follicles have rarely been reported in diseases which are not associated with the presence of autoantibodies (Mackay et al. 1964). Medullary lymphoid follicles have been found in NZB/BL mice, many of which develop spontaneously autoimmune hæmolytic anæmia sometimes associated with a disease resembling systemic lupus erythematosus (Burnet and Holmes 1963).

Only 2 patients with Hashimoto's disease were included in the series. In 1, who was euthyroid, medullary lymphoid follicles were recognised in the thymus, but no thymic abnormality was found in the other patient who had thyrotoxic symptoms; this difference could be explained by the sampling error inherent in small biopsy specimens. The abnormal thymus observed in one case of Hashimoto's disease, however, is similar to that reported in other autoimmune diseases.

The most remarkable finding has been the high incidence of thymic medullary lymphoid follicles in patients with thyrotoxicosis. None of these patients had any of the clinical manifestations of other diseases associated with autoimmunity. While it is possible that the thymic abnormality has been induced by the high level of circulating thyroid hormone or the action of antithyroid drugs, it seems likely that the medullary centres are a manifestation of disordered immunological reactions, and that some cases of thyrotoxicosis may have an autoimmune basis. This has been suggested recently (Anderson 1964), because long-acting thyroid stimulator is associated closely with the gamma-globulin fraction of the serum-proteins (Adams and Kennedy 1962, McKenzie 1962), and it is known to occur in thyrotoxic patients where it is thought to have a pathogenetic role (Adams and Purves 1956, McKenzie 1958, Munro 1959). Furthermore there is considerable clinical evidence of an increased frequency in thyrotoxicosis of other diseases associated with autoimmunity, such as myasthenia gravis (McEachern and Parnell 1948), pernicious anaemia (McNicol 1961), and idiopathic Addison's disease (Blizzard and Kyle 1963, Irvine 1963a). If the hypothesis that some cases of thyrotoxicosis have an autoimmune pathogenesis should prove valid, it might help to explain the high prevalence of antigastric antibodies in both thyrotoxicosis and Hashimoto's disease (Doniach et al. 1963, Irvine 1963b).

The frequency of thymic medullary lymphoid follicles in thyrotoxic patients correlates with the presence of lymphoid follicles in the thyroid but not with thyroid antibodies in the serum. It is therefore tempting to speculate that in these patients thymic medullary lymphoid follicles might be an indicator of cellular immunity of the delayed hypersensitivity type, which is thought to be the



basic pathogenetic mechanism in autoimmune disease (Doniach and Roitt 1962).

### Summary

Using a new technique, the thymus has been biopsied in 103 patients undergoing surgery for thyroid disease.

Thymus medullary lymphoid-follicle formation has been found in association with thyrotoxicosis, and the abnormality can be correlated with the presence of lymphoid follicles in the thyroid.

It has not been possible to correlate these changes with the presence of circulating thyroid antibodies.

This raises the possibility that autoimmunity may be a factor in the pathogenesis of some cases of thyrotoxicosis.

This study was begun after a discussion with Prof. M. F. A. Woodruff, of the department of surgical science, Edinburgh University. We are grateful to Dr. J. Swanson Beck, of the department of pathology, University of Aberdeen, who has supervised the histological and immunological studies and assisted in the preparation of this paper. We are grateful also to Dr. J. S. Brodie, of the City Hospital, Aberdeen, for thyroid autoimmunity tests. One of us (A. G.) was in receipt of a grant from endowment funds through the Board of Management, Aberdeen General Hospitals.

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**RADIOLOGICAL ASSESSMENT OF  
THE THYMUS**

**IN THYROID AND OTHER DISEASES**

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# **RADIOLOGICAL ASSESSMENT OF THE THYMUS IN THYROID AND OTHER DISEASES**

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UNLESS the thymus is grossly enlarged owing to a cyst or tumour, conventional radiographs and tomograms will not reveal any abnormality. The normal or moderately enlarged thymus can only be outlined if gas is used to separate the structures in the anterior mediastinum. Pneumomediastinography was developed by Condorelli (1936) and Lissner (1959), and observations on the thymus in patients with systemic lupus erythematosus have been reported by Hare and Mackay (1963) using this method.

We have assessed the size of the thymus in patients with thyroid disease associated with circulating autoantibodies, and made a comparable study in patients with thyroid disease but in whom no such antibodies could be detected. The size of the thymus has been assessed in patients suffering from other conditions associated with autoimmunity, and in patients in whom pneumomediastinography was used to clarify the nature of an intrathoracic lesion that had been indicated by conventional radiography. Thus we hoped to obtain further knowledge about the role of the thymus in relation to autoimmunity.

## **Patients**

Pneumomediastinography was carried out on 44 patients with thyroid disorders—17 with lymphadenoid goitre (Hashimoto's disease), 18 with thyrotoxicosis, 3 with primary hypothyroidism, 1 with primary hypothyroidism and pernicious anaemia, 1 with primary hypothyroidism and rheumatoid arthritis, 1 with a colloid adenoma and chronic thyroiditis in the remainder of the gland, and 3 with simple goitre.

The clinical diagnosis of thyrotoxicosis and of primary hypothyroidism was confirmed by radioactive-iodine tests, and

estimation of the serum protein-bound iodine. The diagnosis of lymphadenoid goitre was confirmed by needle biopsy or, in one case, by partial thyroidectomy. The patients with simple goitre were studied before partial thyroidectomy, and the clinical diagnosis was confirmed histologically. All patients were euthyroid at the time of the investigation, having been treated with radioactive iodine or antithyroid drugs or thyroxine.

Of the remaining 13 patients, 7 had other disorders that may be autoimmune in nature (2 with myasthenia gravis, 2 with pernicious anaemia, 1 with ankylosing spondylitis, 1 with rheumatoid arthritis, and 1 with idiopathic hypochromic anaemia associated with antinuclear factor), 3 had systemic lupus erythematosus and were under treatment with steroids, and 3 were found to have no disorder that might be expected to influence the size of the thymus—1 with aortic dilatation with negative serological tests for syphilis, 1 with an old tuberculous lesion, and 1 with a parathyroid adenoma.

Patients with large vascular goitres, tracheal compression, upper-respiratory-tract infection, a platelet-count of less than 100,000 per cmm. or disturbed coagulation mechanism were excluded. Where the examination was done for research purposes the object, and details of the procedure, were explained to the patient, and the examination was only carried out if his cooperation was obtained.

Pneumomediastinography was repeated in 1 patient with lymphadenoid goitre after a 3-week course of steroid therapy.

#### Methods

##### *Pneumomediastinography*

After sedation with quinalbarbitone sodium 100–200 mg. and atropine 0.6 mg. intramuscularly, the patient is placed supine with his neck extended. In the retrotracheal technique, a fine straight needle (no. 21) is inserted through the skin immediately below the thyroid isthmus. The needle is carefully advanced through the anterior tracheal wall, and then through the posterior tracheal wall to lie immediately in front of the oesophagus. Frequent aspiration is performed to ensure that the needle has not entered any vascular structure. 100 c.cm. of nitrous oxide is injected with repeated intermittent aspiration under fluoroscopic control using an image intensifier. This is followed by 150–400 c.cm. of oxygen. The patient feels slight discomfort during the introduction of the gas, and this indicates that satisfactory filling of the mediastinum is taking place. The examination causes little distress to the patient. Insufflation is adequate when the gas has reached the diaphragm. A fuller account of the technique is to be published (Sumerling and Irvine 1965).

In patients with large goitres, where the retrotracheal technique may be difficult or impossible, the retrosternal approach may be used. A curved needle (no. 19) is inserted through the skin at the suprasternal notch, and is carefully advanced behind the manubrium. The same volume of gas is injected with the same precautions. This produces good filling



of the anterior mediastinum, but the risk of puncturing vascular structures in the mediastinum may be slightly greater.

In both methods the thymus is outlined by lateral tomography. The maximum cross-sectional area of the thymic shadow in the lateral tomograms is measured by planimetry. The dimensions of the thyroid gland can also be outlined, and this has proved helpful in carrying out needle biopsy of the thyroid in patients with small goitres.

#### Tests for Autoantibodies

The serum of each patient was tested for antibodies to

- Thyroid or gastric antibodies, antinuclear factor, or rheumatoid factor in patient's serum.
- Δ Thyroid or gastric antibodies, antinuclear factor, or rheumatoid factor absent.

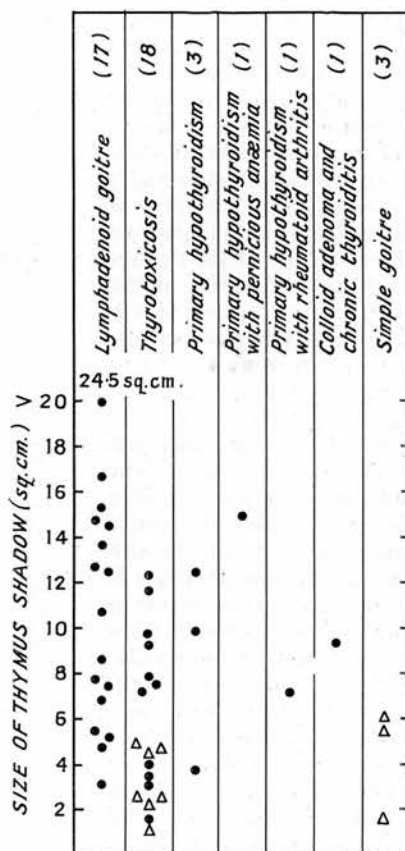


Fig. 1—Cross-sectional area of thymus in 44 patients with various thyroid disorders, with and without autoimmunity.

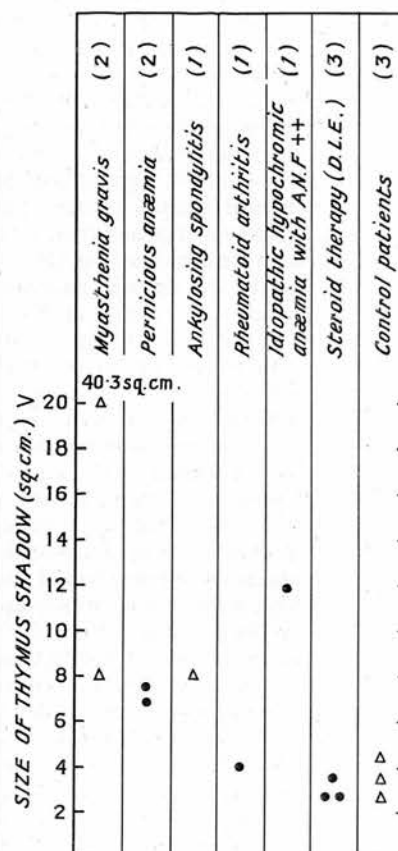


Fig. 4—Cross-sectional area of thymus in 13 patients with non-endocrine diseases with and without autoimmunity.

thyroglobulin by the agar precipitation test and by the method of tanned-cell agglutination (Fulthorpe et al. 1961); to the second colloid antigen (Balfour et al. 1961); to thyroid cytoplasm using the technique of complement fixation (Irvine et al. 1962); to gastric parietal cell cytoplasm using the methods of complement fixation and indirect fluorescent staining (Irvine et al. 1962, Irvine 1963a); and to intrinsic factor (Irvine 1965). The sera were also examined for antinuclear factors using human gastric mucosa and for rheumatoid factor using the differential sheep-cell test. The serum electrophoretic pattern was determined in each patient.

### Results

Fig. 1 summarises the findings in the 44 thyroid patients studied. There is a wide range in the size of the thymus shadow, from 24.5 sq. cm. in a patient with lymphadenoid goitre to 1.0 sq. cm. in a patient with thyrotoxicosis (but no detectable autoantibodies in the serum). The size of the thymus shadow did not exceed 6.0 sq. cm. in any of the 10 thyroid patients who had no serum autoantibodies. 25 of the 34 thyroid patients with autoantibodies had a thymus shadow greater than 6.0 sq. cm. The thymus shadow was greater than 6.0 sq. cm. in 13 of the 17 patients with lymphadenoid goitre. The mean size of the thymus in the 34 thyroid patients with autoantibodies in the serum was 9.3 sq. cm. with a standard deviation of 4.9. The mean size of the thymus in the 10 thyroid patients without autoantibodies was 3.6 sq. cm. with a standard deviation of 1.8 sq. cm. Using Student's *t*-test, this difference is statistically significant ( $P < 0.001$ ).

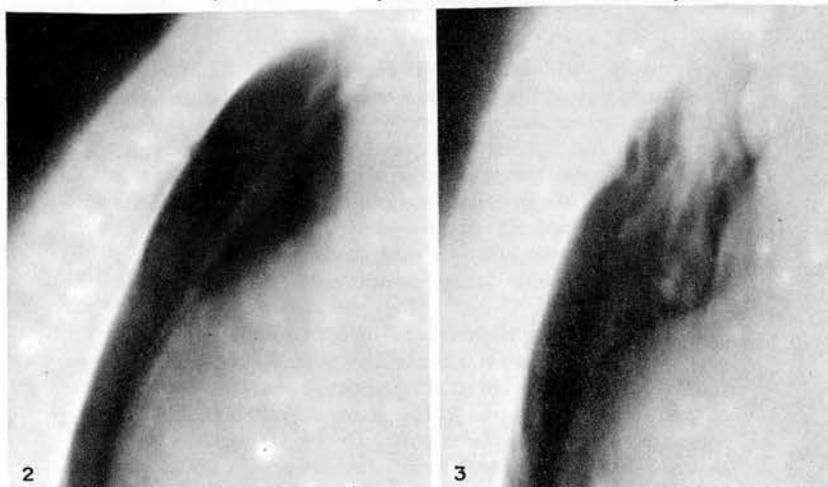
A thymus shadow which is considered to be small and within normal limits (4.4 sq. cm.) is shown in fig. 2. The patient, a woman aged 24, had thyrotoxicosis controlled by carbimazole. An example of an enlarged thymus shadow (13.9 sq. cm.)—in a euthyroid woman of 46 with untreated lymphadenoid goitre—is illustrated in fig. 3. This patient was subsequently given a 3-week course of prednisolone at a dose of 60 mg. per day; the size of her goitre decreased notably, and, on repeat pneumomediastinography, the maximum cross-sectional area of the thymus in the sagittal plane was reduced to 7.5 sq. cm.

The ages of the 44 thyroid patients studied varied from 24 to 65, but no relationship between the age of the patient and the size of the thymus shadow could be found. There was no correlation between the size of the thymus and generalised obesity. Only 2 men with thyroid disease were included in this study: one, aged 51, with lymphadenoid goitre, had very slight thymic enlargement (6.6 sq. cm.); and the other, aged 26, was thyrotoxic, had

autoantibodies in the serum and had a thymus shadow estimated at 4.5 sq. cm.

Fig. 4 shows the findings in relation to thymus size in the 13 other patients studied. The most remarkable of these patients was a man aged 53 with myasthenia gravis of sudden onset. Conventional chest radiography showed no abnormality. After a 3-week period of stabilisation with neostigmine, pneumomediastinography was done and a grossly enlarged thymus (40.3 sq. cm.) was found. Thymectomy was performed 6 weeks later, and a large cystic thymoma was removed. The patient made an excellent recovery, has now returned to work, and requires much less neostigmine than before his operation. This case is illustrated in another paper (Sumerling and Irvine 1965). A second patient with myasthenia gravis was studied. She has had this condition for 15 years with only temporary changes in its severity. The myasthenia was well controlled with pyridostigmine and ephedrine. At pneumomediastinography the maximum cross-sectional area of the thymus shadow was 7.6 sq. cm.

In 2 patients with pernicious anaemia the thymus



**Fig. 2**—Lateral tomogram of chest of a 24-year-old woman with thyrotoxicosis treated with carbimazole.

By pneumomediastinography, the two cornu of a thymus that is judged to be of normal size are demonstrated. This patient had no serological evidence of autoimmunity.

**Fig. 3**—Lateral tomogram of a 46-year-old woman with untreated lymphadenoid goitre confirmed by thyroid biopsy.

An enlarged thymus is demonstrated by pneumomediastinography. The patient's serum was strongly positive for thyroid antibodies.

shadow was shown to be 7.0 sq. cm. in one, and 7.4 sq. cm. in the other. The thymus shadow in a male patient, aged 28, with untreated ankylosing spondylitis was 8.0 sq. cm., and in another male patient aged 32, with rheumatoid arthritis treated by salicylates only, the thymus shadow was 3.8 sq. cm. A woman aged 30, with hypochromic anaemia and strongly positive antinuclear factor, had a thymus shadow of 11.8 sq. cm. The thymus shadow in each of 3 patients who were under treatment with steroids for systemic lupus erythematosus was less than 3 sq. cm.

Fig. 4 includes 3 patients in whom pneumomediastinography was used to study the nature of an abnormal shadow found on conventional radiography, but who had no evidence of autoimmune disorder. One was a man of 65 with aortic dilatation but negative Wassermann reaction and Kahn test; the size of the thymus on X-ray was 4.5 sq. cm. Another was a young man of 26 with a healed tuberculous lesion of some years' standing, and no sign of further tuberculous activity; his thymus shadow was 2.8 sq. cm. The 3rd patient was a woman, aged 55, with a parathyroid adenoma and hyperparathyroidism; her thymus shadow was 3.2 sq. cm.

#### Discussion

According to Hammar (1921) and Boyd (1932) the maximum size of the thymus in man is reached about the 12th year, when its average weight is approximately 35 g. Growth of the thymus then ceases and involution begins. This involution involves only a gradual decline in the actual weight of the organ, although the reduction in the amount of lymphoid tissue—particularly in the cortex but also in the medulla—is more pronounced. If illness occurs, the thymus may temporarily undergo rapid involution.

The separation of the tissues during pneumomediastinography is likely to be no less effective than the dissection of the thymus at operation or at necropsy, but the assessment of the size of the thymus in the present study is limited to measurement in one plane only. In the 10 patients with thyroid disease in whom serum autoantibodies could not be detected, in the 3 control patients, and in the 3 patients receiving steroid therapy, the maximum cross-sectional area of the thymus in the sagittal plane was 6.0 sq. cm. or less. This order of measurement is in keeping with the normal dimensions of the thymus in the adult. There can be little doubt that a cross-sectional area considerably greater than 6.0 sq. cm.—as recorded in many of the



thyroid patients with serum autoantibodies—represents enlargement of the thymus gland. Since no correlation was found between generalised obesity of the individual and the size of the thymus shadow, we infer that at the time of the examination, or previous to it, the amount of lymphoid tissue within the thymus must have been greater than normal, or that the thymus was enlarged owing to the presence of a tumour or cyst. Radiological studies can give no indication of the relative amounts of lymphoid tissue, connective tissue, or fat within the thymus at the time of the examination. The only X-ray that suggested the presence of a thymic tumour or cyst was that of the patient with myasthenia gravis in whom a thymic cyst was later found at operation. The reduction in the size of the thymus shadow in response to steroid therapy in a patient with lymphadenoid goitre accords with the known effect of steroids on the thymus, and presumably the reduction was in the amount of lymphoid tissue within the thymus.

The radiological evidence for thymic enlargement in some patients with thyroid disease, and in patients with myasthenia gravis, is in keeping with earlier histological studies (Sloan 1943, McEachern and Parnell 1948). Gunn et al. (1964) found a correlation between the degree of lymphocytic infiltration within the thyroid and the presence of germinal centres within the thymus. They state that the correlation between the presence of autoantibodies in the serum and histological evidence of thymic abnormalities was poor. Until more is known about the function of the thymus, it cannot be said whether it is an abnormality of the thymus in the past that matters or whether that abnormality has to be continuous in order to account for the persistence of autoimmunity. Thymic medullary lymphoid follicles may be related to cellular immunity of the delayed hypersensitivity type, while generalised hyperplasia of the thymus may be more directly related to the production of self-reacting gamma globulin. Further studies in which the radiological appearances are combined with thymus biopsy are required.

Radiological evidence of thymic enlargement in patients with myasthenia gravis, pernicious anæmia, ankylosing spondylitis, and idiopathic hypochromic anæmia with positive antinuclear factor indicates that disturbance of endocrinological control of the thymus cannot be the only explanation for its enlargement. Some patients with thyroid disease had a history of hyperthyroidism while others had been hypothyroid; all patients were euthyroid

at the time of study. No correlation could be found between the history of thyroid function and the radiological appearance of the thymus. Hare and Mackay (1963) did not consider that there was radiological evidence of thymic enlargement in 2 patients with untreated systemic lupus erythematosus, 1 patient with pernicious anaemia and 1 patient with advanced polymyositis and chronic hepatitis. Kreel et al. (1964) have described a patient with myasthenia gravis and a large thymic shadow who received replacement treatment for hypothyroidism. The normal radiological appearance of the thymus using pneumomediastinography is not yet fully described, and must await improvements in technique and the accumulation of data on patients who have no evidence of immune or endocrine disorder.

Increase in size of the thymus may indicate hyperplasia, either at the time of the investigation or in the past; it does not necessarily mean that the gland is hyperactive. Compensatory hyperplasia causing an enlarged thymus may be a response to some inherent abnormality, but the overall effect may be a deficiency of thymic function. Sutherland et al. (1965) have reported that a positive Coombs test commonly develops in rabbits after neonatal removal of the thymus, and appendix. In the NZB/BL strain of mice that develop autoimmune disease spontaneously, neonatal thymectomy does not prevent the development of the autoimmune process (Helyer and Howie 1963). In man, systemic lupus erythematosus and lymphadenoid goitre have been found to develop after thymectomy for myasthenia gravis (Alarcón-Segovia et al. 1963, Simpson 1964). Adner et al. (1964)—in a study of 48 patients with myasthenia gravis—found a high frequency of antibodies against normal tissue, and a reduced ability to express delayed (cellular) hypersensitivity reactions. While there is evidence that thyroid autoimmunity may result from impaired immunological tolerance (Irvine 1964), these observations on myasthenia by Adner et al. have still to be reconciled with the idea that cellular immunity of the delayed hypersensitivity type is probably the basic pathogenic mechanism in thyroid autoimmune disorder (Doniach and Roitt 1962, Irvine 1963b).

#### Summary

Pneumomediastinography was carried out on 44 patients with various thyroid disorders. In 10 of these patients there was no serological evidence of autoimmunity, and the maximum size of the thymic shadow in the sagittal

plane varied from 1.0 to 6.0 sq. cm. In 25 of 34 patients with autoimmunity the thymic shadow was larger than 6.0 sq. cm. The degree of thymic enlargement in thyroid disease associated with autoimmunity is statistically significant.

Radiological evidence of thymic enlargement was also observed in 2 patients with myasthenia gravis, 2 with pernicious anaemia, 1 with ankylosing spondylitis, and 1 with idiopathic hypochromic anaemia and a positive test for antinuclear factor. The thymus was of normal size in 3 patients without thyroid disease or serological evidence of autoimmunity.

The possible significance of thymic enlargement in relation to autoimmunity is discussed.

We are grateful to our clinical colleagues for their cooperation and to Mr. Kenneth Marwick, Miss Laura Scarth, and Miss Irene Park for their technical and secretarial assistance.

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**PNEUMOMEDIASTINOGRAPHY**

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### **Pneumomediastinography [Summary]**

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The technique of pneumomediastinography is not well known in this country but has been used in Europe for many years. A percutaneous method is the technique of choice and we have preferred Condorelli's retrotracheal method (Condorelli 1936, Condorelli *et al.* 1951). One of us (M D S) was fortunate in learning the technique from Dr J Lissner at Frankfurt (*see* Lissner 1959).

The patient is fasted and sedated, and under local anaesthesia a fine needle (No. 21 SWG) is introduced through the trachea into the retrotracheal space; 300-500 ml of gas is injected slowly under fluoroscopic control. A series of tomograms in the anteroposterior and lateral planes show stripping of the pleura off the peri-

cardium, and the superior vena cava, aorta, great vessels, pulmonary artery, azygos vein and the thymus are demonstrated. The outer walls of the main bronchi are outlined and on occasions the ligamentum arteriosum is visible.

In some patients with gross thyroid enlargement the retrosternal method of inserting the needle behind the manubrium over the suprasternal notch has been used. Kreel *et al.* (1964) have also described a transsternal approach.

Ninety-two patients have been investigated, and the examinations have been followed by no morbidity. The technique has been used largely to demonstrate the size and shape of the thymus, particularly in relation to thyroid disease and autoimmunity (Irvine & Sumerling 1965).

In a series of 44 patients with thyroid disease, radiological evidence of thyroid enlargement was frequently found in patients with auto-antibodies in the serum and not in those with negative serology.

Fig 1 is an example of a thymus shadow that is considered to be within normal limits, while in Fig 2 the thymus is markedly enlarged.



Fig 1 Lateral tomogram of chest of a 38-year-old euthyroid woman treated by partial thyroidectomy for thyrotoxicosis three years previously. The thymus gland is considered to be within normal limits

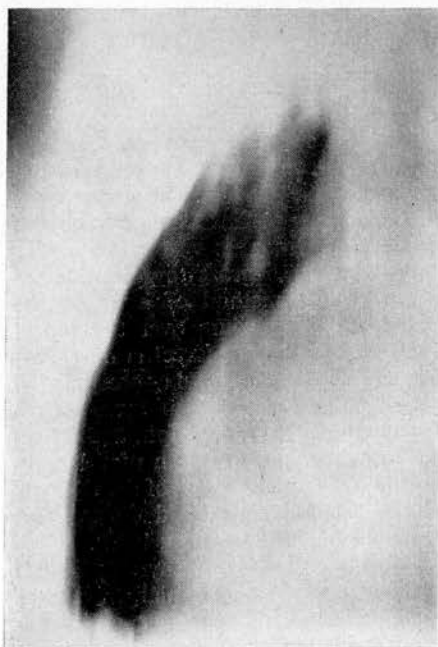


Fig 2 Lateral tomogram of chest of a 37-year-old euthyroid woman with untreated lymphadenoid goitre (Hashimoto thyroiditis). The thymus gland is markedly enlarged

In 12% of patients with myasthenia gravis thymic tumours may be discovered at operation (Keynes 1949). In a man of 55 with myasthenia gravis pneumomediastinography revealed a large mass in the anterior mediastinum, and at operation a large thymic cyst was removed. Similar cases have been described by Hare & Mackay (1963) and by Kreel *et al.* (1964).

Masses in the mediastinum may not always be demonstrated by conventional methods and may be missed if they do not alter the radiographic contours. A man of 55 who had lymphosarcoma of cervical and axillary glands showed no abnormality in conventional X-ray films and tomograms, but the investigation revealed enlarged mediastinal glands. It is anticipated that small glands of 1–2 cm diameter may be detected by the technique.

The chest X-ray of a woman aged 55 with suspected hyperparathyroidism demonstrated a mass lying in the superior mediastinum. This mass simulated an aneurysm of the innominate artery, but the outlining of the mass by gas showed that it was a separate tumour lying adjacent to the innominate artery. At operation a parathyroid adenoma with cystic degeneration was excised. Posen *et al.* (1964) have described a similar case, but in their patient the mass was not demonstrated on the conventional X-ray films.

A preliminary investigation was made to determine the value of the technique in assessing the operability of carcinoma of the bronchus, particularly at the left hilum. In the absence of lung disease, gas diffuses between the aorta and the pulmonary artery and along the main bronchi. In a patient with inoperable carcinoma (left phrenic and left recurrent palsies were present) there was failure of dissection and a plaque of growth was shown at the left hilum. In a second patient with a large mass at the left hilum gas diffused between the aorta and the main pulmonary artery down to the left pulmonary artery, and the ligamentum arteriosum was shown. The aortic window and the superior mediastinum were shown to be free of glandular involvement. These findings were confirmed at operation when a successful pneumonectomy was performed.

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**PNEUMOMEDIASTINOGRAPHY**

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**Amer. J. Roentgenol (1966), 98, 451-460.**



## PNEUMOMEDIASTINOGRAPHY\*

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THE study of the mediastinum by conventional roentgenography is limited because the component tissues have similar densities. The internal lumen of the various vascular structures of the mediastinum may be demonstrated by the techniques of venography, angiocardiology, aortography and intraosseous venography, and these methods have all been employed in the investigation of mediastinal pathology. The lumen of the esophagus may be outlined by barium sulfate paste and the trachea and bronchi may be studied by the techniques of tomography and bronchography.

Condorelli developed 2 methods for the demonstration of the mediastinal structures by the injection of gas into the mediastinum; the retrosternal technique<sup>4</sup> and the transtracheal approach.<sup>5</sup>

## METHOD

## TRANSTRACHEAL

Straight roentgenograms of the chest and thoracic inlet are made to ensure that there is no untoward compression or deviation of the trachea. The patient is fasted before the examination and sedated with a short acting barbiturate (cyclobarbitone 100–200 mg.) together with atropine sulfate 0.6 mg. intramuscularly. The neck is hyperextended over a pillow and the skin and subcutaneous tissues either just above or below the thyroid isthmus, are infiltrated with 1 per cent lignocaine solution. The lower route just below the thyroid isthmus is preferred, but if there is a prominent aortic arch the higher route above the thyroid isthmus is used. Infiltration is continued down to the trachea and the needle is advanced between 2 tracheal rings into the

lumen of the trachea. Two cubic centimeters lignocaine solution are then injected into the trachea. The patient will cough at this stage, indicating the correct positioning of the needle. The needle is withdrawn and a child's lumbar puncture needle (size 21) is inserted along the same route into the trachea. The needle is then further advanced until resistance to continuous aspiration proves that the point of the needle has just passed through the posterior tracheal wall. Gas is then injected in aliquots of 50 cc., drawn into a syringe from a cylinder through a three-way tap. Aspiration before injection ensures that the needle has not entered a blood vessel inadvertently. Nitrous oxide is employed for the first 100 cc., and if dissection by the gas proceeds normally, this is followed by 200–400 cc. of oxygen. Nitrous oxide or carbon dioxide used alone is absorbed too rapidly and if further roentgenograms are required most of the gas may have been absorbed. The progress of the examination is checked by frequent fluoroscopic control—using a Philips television 9 inch intensifier system. During the induction, the patient experiences some slight discomfort of the chest and on auscultation characteristic crepitations may be heard. No changes have been observed in the electrocardiogram, pulse rate or blood pressure taken at intervals during the insufflation. A satisfactory examination is indicated by the separation of the mediastinal pleura from the pericardial sac down to the level of the diaphragm. Linear tomograms are taken in lateral and anteroposterior projections. In a patient of 60 kg. the exposure factors would be 75 kv. and 100 mas. for anteroposterior roentgenograms and 90 kv. and 160 mas. for lateral

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roentgenograms. When the thymus is to be delineated, less exposure is required: 72 kv. and 100 mas. for the anteroposterior tomograms and 75 kv. and 100 mas. for the lateral tomograms. Following the examination, the patient is confined to bed for 6 hours.

In this method, there is good filling of both the anterior and posterior compartments of the mediastinum and the patient experiences little discomfort.

#### RETROSTERNAL

In some patients the transtracheal method was contraindicated because of thyroid enlargement sometimes with tracheal deviation or compression. In these cases the retrosternal method was used. A fine curved needle (size 20) is inserted percutaneously through the suprasternal notch so as to just enter the anterior mediastinum behind the manubrium. Injection of the gas will lead to good filling of the anterior mediastinum but filling of the posterior mediastinum is less satisfactory. Discomfort is minimized by introducing the gas into the anterior mediastinum slowly. In the retrosternal method there is theoretically a greater risk of encountering a vascular structure but in our experience this complication has not occurred.

Using the above techniques, pneumomediastinography by either the transtracheal or retrosternal method has not been followed by any morbidity. Stringent precautions were taken against the possibility of gas embolism. The examination must not be undertaken if there is any evidence of upper respiratory tract infection and full asepsis must be maintained. The examination is contraindicated in patients with a bleeding diathesis. With careful positioning, the esophagus should not be punctured during the retrotracheal method. This may have occurred on one occasion in the present series but with no ill effect.

#### CLINICAL OBSERVATIONS

The examination of pneumomediastinography has been carried out in 110 patients

suffering from a variety of conditions. For the present paper, a number of cases of particular interest has been selected in order to illustrate and discuss the application of this technique.

#### NORMAL APPEARANCES

The appearance of the "normal" mediastinum as studied by pneumomediastinography is shown in Figures 1 and 2.

In the anteroposterior view, the gas outlines the left innominate vein and the superior vena cava, which tends to move to the right. The azygos vein may be seen in the right tracheobronchial angle. The aorta, innominate artery, left carotid artery and left subclavian artery can readily be demonstrated in a series of anteroposterior

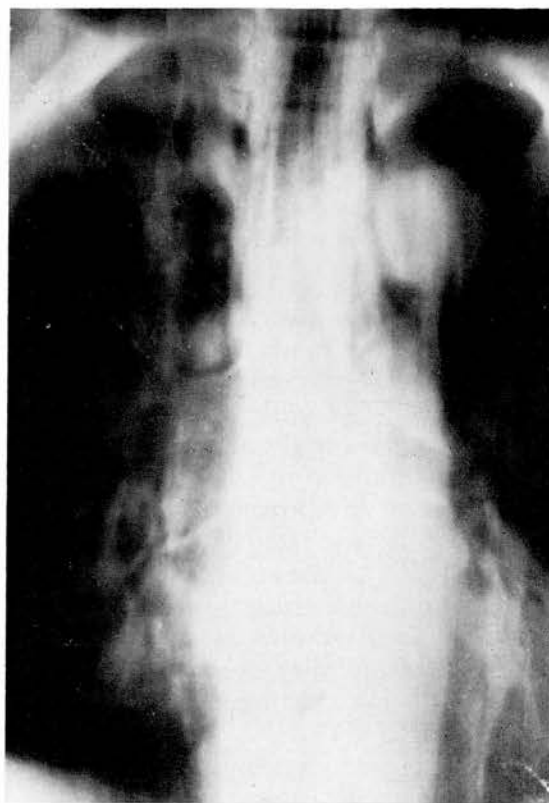


FIG. 1. Anteroposterior tomogram showing normal anatomy. Gas has outlined the aorta, main pulmonary artery, ligamentum arteriosum, azygos vein, left subclavian artery and superior vena cava. Gas also diffused around the trachea and main bronchi, outlining their walls.

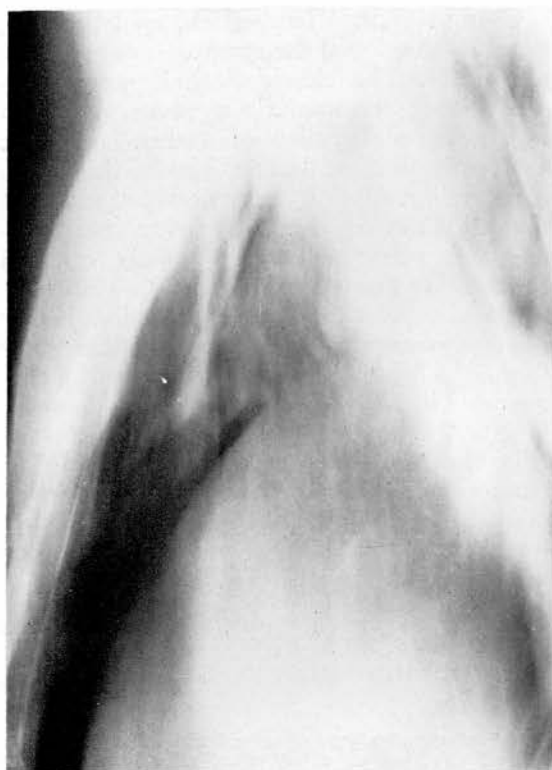


FIG. 2. Lateral tomogram showing the two lobes of the thymus outlined by gas in the anterior mediastinum. The trachea, esophagus, left innominate vein and superior vena cava are also seen. Gas has diffused between the right ventricular outflow tract and the aorta.

tomograms. When sufficient gas has been injected into a patient with a normal mediastinum, dissection will occur around the main pulmonary artery and aortic arch, outlining the aortic window (Fig. 1). The outer walls of the trachea and bronchi can be outlined as far as the primary divisions and in many cases the ligamentum arteriosum can be identified. The thymus may be distinguished in the appropriate anteroposterior tomogram as 2 thin shadows on either side of the mid-line. The right lobe of the thymus is closely related to the superior vena cava.

The lateral tomograms following the insufflation of gas are particularly suitable for outlining the thymus. The 2 lobes of what is considered to be a normal thymus are demonstrated in Figure 2. The thymus

may be seen roentgenologically to extend a variable distance inferiorly in the anterior mediastinum. Also, in the lateral tomograms the left atrial wall may be clearly visualized and the external surface of the esophagus may be demonstrated.

During pneumomediastinography, the gas diffuses into the neck and anteroposterior tomograms will clearly delineate the lobes of the thyroid gland (Fig. 3).

#### DISORDERS OF THE THYMUS

An assessment of the size of the thymus can be made by measuring with the aid of a planimeter the area of the thymic shadow in the sagittal tomogram. Three examples of thymic disorder are described below.

CASE 1. J.B. This 56 year old woman presented with non-goitrous idiopathic primary hypothyroidism. The clinical diagnosis was confirmed by a low serum level of protein bound



FIG. 3. Anteroposterior tomogram of the cervical region shows the two lobes of the thyroid gland outlined by gas immediately below the larynx.



FIG. 4. Case I. Lateral tomogram. Moderately enlarged thymus in a patient with primary hypothyroidism and whose serum contained thyroid antibodies.

iodine (P.B.I. =  $1.5 \mu\text{g}$ . per cent) and a low thyroid gland uptake of  $\text{I}^{131}$  following an oral dose of  $20 \mu\text{c}$  (48 hr. uptake = 0.9 per cent). Pneumomediastinography was performed after 4 months replacement treatment with thyroxine, 0.2 mg. per day. The thymus is considered to be diffusely enlarged with a cross-sectional area in the sagittal tomogram of  $13 \text{ cm}^2$  (Fig. 4). At the time of the roentgenographic examination, the patient's serum contained antibody to thyroglobulin in a titer of 1:250 using the tanned cell hemagglutination technique.<sup>6</sup>

A possible relationship between roentgenologic evidence of hyperplasia of the thymus in patients with thyroid disease associated with auto-immunity has been the subject of a separate paper.<sup>11</sup> It was observed that abnormally large thymic shadows were a common occurrence in patients with thyroid disease and who had organ-specific antibodies demonstrable in the serum.

CASE II. W.B. A lumberjack, aged 53 years, suddenly developed the symptoms of myasthenia gravis. The classic clinical picture was confirmed by the dramatic improvement following the administration of anticholinesterase. Conventional chest roentgenograms showed no abnormality. After a 3 week period of stabilization with neostigmine, pneumomediastinography was performed and a grossly enlarged thymus was demonstrated (Fig. 5). The cross-sectional area of the thymus shadow in the sagittal tomogram was  $40.3 \text{ cm}^2$  and the shape of the shadow indicated the presence of a thymoma. Thymectomy was performed 6 weeks later and a large cystic thymoma was removed (Fig. 6). The cyst contained 35 ml. of fluid. The patient made a good recovery and for a period of some months his requirement for neostigmine was low, but subsequently large doses were again necessary to control his myasthenia.

This case illustrates that conventional chest roentgenograms may not always



FIG. 5. Case II. Lateral tomogram. Myasthenia gravis of recent onset. Gas has outlined a clearly defined large mass lying immediately in front of and to the side of the aorta in the anterior mediastinum.

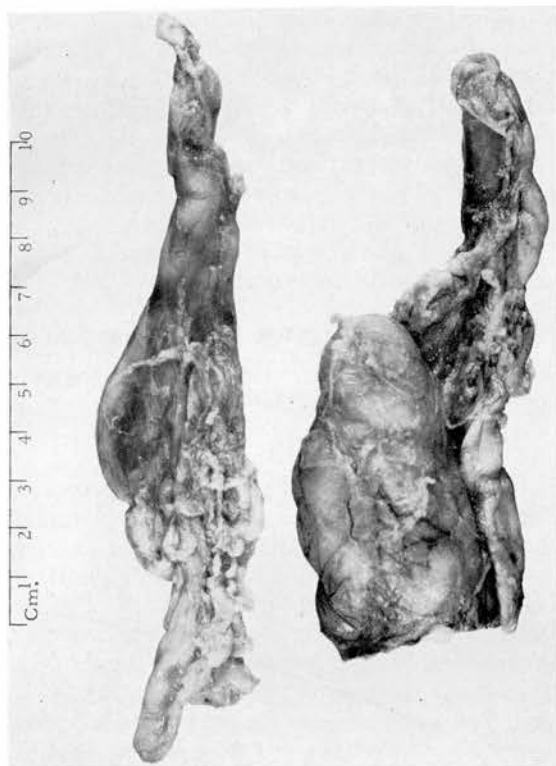


FIG. 6. Case II. Operative specimen (cut longitudinally into 2 parts). The thymus is considerably enlarged due to the presence of a large cyst, the wall of which contained thymoma tissue.

demonstrate thymic enlargement even when this takes the form of a thymoma of considerable size. The thymoma was readily demonstrated at pneumomediastinography.

CASE III. C.B. This 26 year old woman presented with an 8 month history of a dry irritating cough and breathlessness on severe exertion. A conventional roentgenogram of the chest showed a mass in the superior mediastinum and the possibility of an aortic aneurysm was considered by the referring physician. A scintigram ( $I^{131}$ ) showed a normal distribution of thyroid tissue. Pneumomediastinography using the retrosternal method showed that there was a large lobulated mass lying in the anterior mediastinum closely applied to the aorta and in close relationship to the pulmonary artery. Gas failed to separate the mass from the aorta (Fig. 7 and 8). The mass extended across the mid-line as far as the right border of the sternum. There was no evidence of thymic tissue apart from the mass and it was, therefore, concluded that the

mass was either a tumor or cyst arising from the thymus or that the thymus was the site of some other pathology, *e.g.*, reticulosis. There was no evidence of any other mass or of glandular involvement elsewhere in the mediastinum.

At thoracotomy, a large lobulated mass was found in the anterior mediastinum adherent to the aorta. Most of the mass was removed but a small part of the tumor could not be removed because of infiltration into the aorta. Histologic examination showed the tumor to be a lymphadenoma and although no thymic tissue could be identified the tumor was presumed to have arisen in the thymus.

Pneumomediastinography in this patient demonstrated that the mediastinal mass was adherent to the aorta, and, in retrospect, one might have concluded that the tumor was inoperable and that the patient might have been treated by radiotherapy alone.



FIG. 7. Case III. Lateral tomogram. Lymphadenoma possibly arising in thymus. The gas has outlined a mass lying in the anterior mediastinum anterior to the ascending arch of the aorta. Gas failed to dissect between the aorta and the mass and at operation the mass was adherent to the ascending arch, preventing complete removal.





FIG. 8. Case III. Anteroposterior tomogram (same patient as in Figure 7) shows an extensive mass extending across the whole anterior mediastinum. Gas fails to pass between the mass and the surrounding structures.

#### PARATHYROID ADENOMA

CASE IV. M.R. This 55 year old woman presented with urinary infection. On investigation, she was found to have a large stag horn calculus in the pelvis of the right kidney. The serum  $\text{Ca}^{++}$  was 12.2 mg. per cent, serum inorganic phosphate was 1.92 mg. per cent and the serum alkaline phosphatase was 9.5 units. Examination of the neck showed 2 nodular discrete swellings in the region of the thyroid, but a scintigram ( $\text{I}^{131}$ ) showed normal distribution of functioning thyroid tissue. A chest roentgenogram showed a rounded mass in the superior mediastinum on the right side. The mass was seen to be situated mainly behind the trachea and to the right of the esophagus. The roentgenologic diagnosis rested between an aneurysm of the innominate artery and a tumor mass lying in the anterior mediastinum. Pneumomediastinography clearly demonstrated the rounded mass in the right of the superior mediastinum and showed it to be situated lateral to the trachea and posterior to the superior vena

cava and separate from the innominate artery (Fig. 9). There was no evidence of aberrant parathyroid tissue in the anterior mediastinum. Some enlargement of the right lobe of the thyroid was demonstrated.

The neck was explored and an enlarged lower parathyroid gland was found within the upper mediastinum and removed. Histologically, the parathyroid gland was adenomatous and had undergone cystic degeneration.

This case illustrates the application of pneumomediastinography to the investigation of parathyroid adenoma.

#### MEDIASTINAL LYMPHADENOPATHY

CASE V. A.C. This 62 year old man presented with bilateral cervical and axillary lymphadenopathy of undetermined duration. Cervical lymph node biopsy established a diagnosis of lymphosarcoma. Although conventional roentgenograms of the chest showed no abnormality, mediastinal lymphadenopathy was clearly demonstrated at pneumomediastinography (Fig. 10). Ten months later he required abdominal

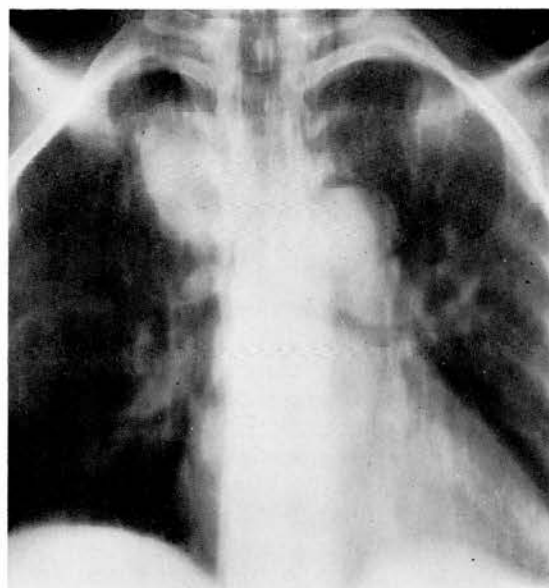


FIG. 9. Case IV. Anteroposterior tomogram. Hyperparathyroidism. Large mass in the right superior mediastinum outlined by gas. The azygos vein and aortic knuckle are also shown. In other tomographic cuts, the innominate artery could be identified medial to the mass. At operation, a parathyroid adenoma with cystic degeneration was excised.



FIG. 10. Case v. Anteroposterior tomogram of a patient with lymphosarcoma of cervical and axillary lymph nodes. Gas has outlined an enlarged lymph node in the right superior mediastinum. There are probably 2 other small lymph nodes present, 1 in each hilus.

radiation therapy following the development of a central abdominal lymphoid mass.

This case illustrates that conventional roentgenography may fail to demonstrate mediastinal lymphadenopathy. The use of pneumomediastinography may enable more accurate assessment of tumors of the reticulo-endothelial system.

#### CARCINOMA OF BRONCHUS

CASE VI. W.C. A male patient, aged 63 years, with a carcinoma of the left main bronchus had evidence of left recurrent laryngeal and left phrenic palsy, together with a Horner's syndrome. The clinical evidence indicated an inoperable tumor and pneumomediastinography revealed a failure of dissection of the gas around the left hilus and the pulmonary artery. A linear opacity was demonstrated alongside the mediastinum at the level of the left hilus

and this was presumed to be a plaque of neoplastic tissue (Fig. 11).

CASE VII. J.B. This male patient, aged 63 years, was admitted complaining of a 2 month history of hemoptysis. A chest roentgenogram demonstrated a large opacity in the anterior segment of the left upper lobe. Pneumomediastinography showed that gas had passed along the main pulmonary artery and into the aortic window. The first part of the left pulmonary artery was also outlined by gas. The ligamentum arteriosum could be identified (Fig. 12). The aortic window was free of enlarged lymph nodes and no enlarged lymph nodes were demonstrated elsewhere in the mediastinum. At operation a solid, firm neoplasm, 5 cm. in diameter, was found near the hilus, surrounding the upper lobe bronchus. The tumor had extended along the left pulmonary artery to a point 1 inch distal to the ligamentum arteriosum. There

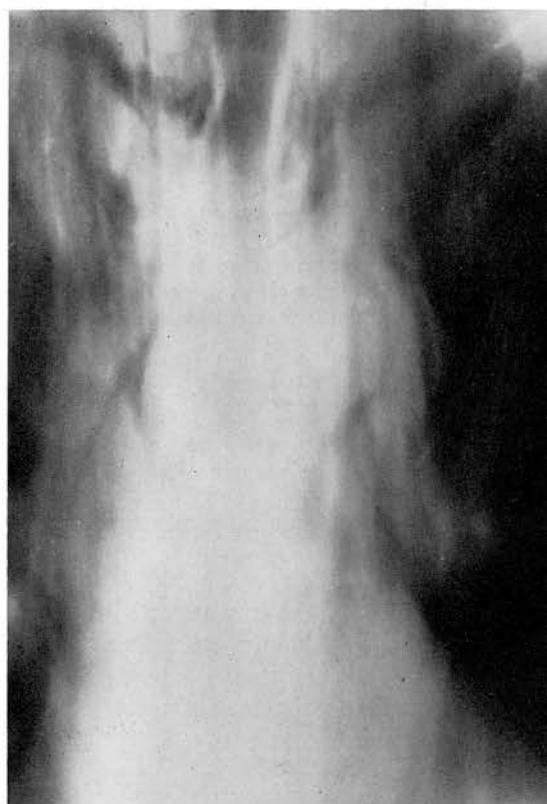


FIG. 11. Case vi. Anteroposterior tomogram. Inoperable carcinoma of the left bronchus. Gas failed to diffuse between the main pulmonary artery and the aorta and a mass is present adjacent to these structures—? plaque of neoplastic tissue.

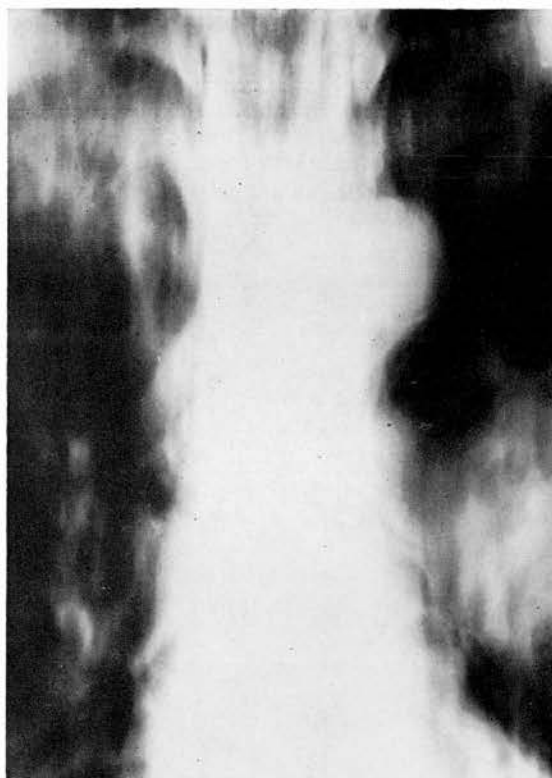


FIG. 12. Case VII. Anteroposterior tomogram. Large malignant mass arising from the left main bronchus. Gas diffuses between the aorta and the main pulmonary artery as far as the left pulmonary artery. The ligamentum arteriosum can be identified just internal to the pleural reflection. The lung containing the tumor was removed without difficulty.

was no involvement of the mediastinum. A pneumonectomy was performed without difficulty. On histologic examination, the tumor was found to be a squamous cell carcinoma, and it had infiltrated to a point 1 cm. distal to the divided bronchus.

These 2 cases (Case VI and VII) illustrate the possibility of using pneumomediastinography to determine the operability of bronchial carcinoma, particularly when left hilar involvement is questioned.

#### DISCUSSION

Condorelli<sup>4</sup> in 1936 published his paper on the retrosternal method of pneumomediastinography. This method succeeds in producing adequate filling of the anterior

mediastinum and is the technique which we have favored [for the outlining of the thymus. Marchand,<sup>17</sup> from fluid injection studies in autopsy material, concluded that fascial sheaths enclosed the great vessels, esophagus and trachea, and that these prevent the free circulation of fluid injection into the mediastinum. In common with Hughes *et al.*<sup>9</sup> we have found that the mediastinum behaves differently when gas is injected. Gas tends to diffuse widely through the mediastinum and also upwards into the neck. After injection of gas into the anterior mediastinum using the retrosternal method, gas will pass into the posterior mediastinum, particularly if the patient is turned into the prone position. However, filling of the posterior mediastinum is not so adequate compared with the transtracheal method.

Other techniques of pneumomediastinography include the injection of gas through a needle inserted directly through the manubriosternal joint into the anterior mediastinum.<sup>14</sup> Baccaglini,<sup>1</sup> Betoulières *et al.*,<sup>3</sup> Isard *et al.*,<sup>12</sup> and Tapiovaara<sup>20</sup> used the presacral method of insufflation, allowing the gas in the retroperitoneal space to pass upwards into the mediastinum. This method was employed because the danger of gas embolism was considered to be less than in the direct approaches. According to Hughes *et al.*,<sup>9</sup> the presacral method is unreliable and in our experience a large amount of gas must be injected, producing much discomfort. Šimeček and Holub,<sup>19</sup> while investigating patients with suspected bronchial carcinoma, injected gas through a needle inserted through the tracheal wall during bronchoscopy. Berne *et al.*<sup>2</sup> performed routine scalene lymph node biopsies in patients with suspected bronchial carcinoma, and as an extension of the procedure they injected gas through a catheter introduced into the mediastinum through the operation site. When the retrosternal technique could not be used because of masses lying in the thoracic inlet, Hughes *et al.*<sup>9</sup> inserted a needle upwards into the anterior mediastinum behind the xiphisternum.



In our experience the retrosternal method gives satisfactory delineation of the thymus although the technique cannot distinguish between the relative proportions of lymphoid tissue, fat and connective tissue within the thymus. In a series of 44 patients with thyroid disease, roentgenologic enlargement of the thymus was frequently noted in those who had evidence of auto-immunity.<sup>11</sup> The retrosternal method was used by Hare and Mackay<sup>7</sup> in the investigation of 12 patients with other disorders associated with auto-immunity.

Keynes,<sup>13</sup> in a series of 155 patients with myasthenia gravis subjected to thoracotomy, found that 11.6 per cent had tumors in the thymus. Kreel, Blendis and Piercy<sup>14</sup> believe that it is important to recognize tumors preoperatively in order to plan the correct treatment. Pneumomediastinography will clearly outline thymic tumors as in the case reported in this paper. Harper and Guyer<sup>8</sup> believe that tumors of the thymus should always be delineated by tomograms taken at suitable depths and with suitable projections. Kreel<sup>15</sup> has been able to demonstrate small tumors by pneumomediastinography in 2 patients with severe myasthenia gravis where straight roentgenograms and tomograms revealed no abnormality. A similar case is described in the present paper. If a thymic tumor does not encroach on the mediastinal borders, it is unlikely to be visualized by conventional roentgenography.

Posen *et al.*<sup>18</sup> described a patient with primary hyperparathyroidism who had normal conventional roentgenograms, but on pneumomediastinography a mass was demonstrated in the superior mediastinum, and this proved to be a parathyroid adenoma at operation. In Case IV of the present paper, a parathyroid adenoma was clearly delineated at pneumomediastinography and at subsequent operation a parathyroid adenoma was removed. The clear demarcation of the thyroid at pneumomediastinography together with I<sup>131</sup> scintiscans also helps to resolve the differential diagnosis of a superior mediastinal mass

seen by conventional roentgenography. Delineation of the thyroid by gas may also be of value in providing an objective measurement of thyroid size and be helpful in performing a needle biopsy of the thyroid.

In Case V of the present paper, the chest roentgenograms were negative in a patient with lymphosarcoma involving cervical and axillary lymph nodes. At pneumomediastinography, enlarged mediastinal lymph nodes were clearly demonstrated. This finding is of some importance in determining the grading of the disease, its treatment and its prognosis. It is anticipated that lymph nodes of 1 cm. in diameter might be identified by this means.

While the diagnosis of carcinoma of the bronchus may be established by the use of techniques including roentgenology, bronchoscopy and possibly lung biopsy, the possibility of a successful resection is much more difficult to determine before thoracotomy. While venography and angiography may determine, to some extent, the degree of lymph node involvement and mediastinal spread, these methods are limited. Only lymph nodes that lie close to the superior vena cava will cause distortion of that structure. Bronchial arteriography will not necessarily show up all the lymph nodes involved and may not indicate the extent of the spread of the tumor. Thoracic surgeons have particular difficulty in assessing the spread of growth from a left hilus tumor towards the mediastinum. Šimeček and Holub,<sup>19</sup> using the pertracheal method during bronchoscopy, examined 500 patients with carcinoma of the bronchus and found the method of considerable value in assessing involvement of the mediastinal lymph nodes and the extent of mediastinal infiltration. They claim to be able to exclude patients who have inoperable tumors from unnecessary thoracotomy and from the roentgenologic appearances claim that they can differentiate between enlargement of mediastinal lymph nodes due to tumor from those due to inflammation. Ikins *et al.*<sup>10</sup> introduced a catheter through the operation wound following



scalene lymph node biopsy. Large amounts of carbon dioxide, from 1 up to 8 liters, were injected into the mediastinum. In a series of 31 patients with bronchial carcinoma, they found the method of considerable value in assessing the degree of lymph node metastases and mediastinal infiltration. We have performed insufficient examinations to assess fully the usefulness of the method but believe that pneumomediastinography has a place in the investigation of patients with carcinoma of the bronchus with a view to excluding patients with inoperable carcinoma from unnecessary thoracotomy.

## SUMMARY

Pneumomediastinography is a safe procedure provided the technique is carried out with proper precautions against gas embolism. The transtracheal and retrosternal methods of Condorelli<sup>4</sup> have been performed on 110 patients without morbidity.

The method has been employed for the investigation of the thymus, the parathyroid glands, mediastinal lymphadenopathy and the thyroid gland. The possibility of using the method to assess operability of bronchial carcinoma is discussed.

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CHAPTER IX

CHAPTER IXSTUDIES ON AUTOIMMUNITY IN EXPERIMENTAL ANIMALS

"Studies on Experimental Autoimmune Thymitis in Guinea-pigs".

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Studies on experimental autoimmune thymitis in guinea-pigs

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AND W. J. IRVINE

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## STUDIES ON EXPERIMENTAL AUTOIMMUNE THYMITIS IN GUINEA-PIGS

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(Received 19 April 1969)

### SUMMARY

Normal and thymectomized outbred guinea-pigs were immunized with extracts of calf thymus, calf skeletal muscle or calf heart muscle emulsified in Freund's complete adjuvant. Immunization with either thymus or skeletal muscle produced a significant incidence of thymitis and a partial neuromuscular block in contrast to control animals either untreated or injected with an extract of calf lymph node or saline in Freund's complete adjuvant. The incidence of thymitis and partial neuromuscular block in animals that were injected with an extract of calf heart muscle in Freund's complete adjuvant was found not to be significant when compared to the control animals.

Neuromuscular transmission was studied using Copeland-Davis clip electrodes or a bipolar silver wire electrode threaded through the flexor digitorum muscle. The development of partial neuromuscular block in the test animals was found to be dependent on the presence of the thymus. All animals with a partial neuromuscular block had evidence of experimental thymitis.

These findings are in keeping with the hypothesis that a factor released by the thymus may be important in the development of the neuromuscular block characteristic of myasthenia gravis.

### INTRODUCTION

The thymus gland in myasthenia gravis has one of three histological appearances (Castleman, 1966). About 10% of patients have thymomas. Of the remaining patients, the thymus is normal macroscopically and microscopically in 20% while in the other 80% germinal centres are conspicuously present in the thymic medulla. In about 35% of myasthenia patients without a thymoma and in approximately 95% of patients with associated thymoma, serum autoantibody can be demonstrated reacting with striations of skeletal muscle and heart muscle and with the cytoplasm of the thymus myoid cells (Strauss *et al.*, 1965). In attempts

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to produce similar histological appearances and antibodies with similar reactivity in experimental animals, Marshall & White (1961) reported that the formation of germinal centres in the thymic medulla could be produced by intrathymic injection of a typhoid-paratyphoid vaccine or diphtheria toxoid. Namba & Grob (1966) succeeded in raising skeletal muscle antibodies in rabbits by the repeated injections of a human skeletal muscle ribonucleoprotein in Freund's complete adjuvant (CFA). Goldstein & Whittingham (1966, 1967) and Goldstein & Hofman (1968) showed that guinea-pigs or rats injected with heterologous or homologous thymus or skeletal muscle in CFA developed a thymitis, serum antibodies to skeletal muscle and thymus myoid cells and also a partial neuro-muscular block. In contrast to these positive findings, negative results in similar experiments have been reported by Strauss (1963) and by Parkes (1966).

This paper describes our own experience in the attempt to produce an experimental model of myasthenia gravis in guinea-pigs.

## MATERIALS AND METHODS

### *Animals*

Outbred guinea-pigs of both sexes, 4-6 months old were used.

### *Antigen preparation*

Fresh thymus, skeletal muscle, heart muscle and lymph node from a young calf were homogenized in phosphate buffered saline, pH 7.2 (20% w/v). The homogenates were centrifuged for 15 min at 5000 rev/min and the supernatant emulsified in an equal volume of Freund's complete adjuvant (CFA).

TABLE 1. The number of animals injected with a given antigen subdivided according to the two methods used for electromyography and whether or not thymectomy had previously been done

No. of animals		Treatment
Thymus intact	Thymectomized	
Group A		
10		Calf thymus + CFA
10		Calf skeletal muscle + CFA
4		Calf lymph node + CFA
Group B		
9		Calf thymus + CFA
8		Calf skeletal muscle + CFA
9		Calf heart muscle + CFA
5		Calf lymph node + CFA
8		Saline + CFA
10		No treatment
	7	Calf thymus + CFA
	8	Calf skeletal muscle + CFA
	4	Calf lymph node + CFA

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### Immunization

Each animal was injected with 0.1 ml antigen in CFA in each hind footpad. The number of animals injected with a given antigen is as shown in Table 1. The animals were subdivided into two groups A and B according to the two different methods used for electromyography. In addition five thymectomized animals were immunized with 0.1 ml of a crude guinea-pig <sup>thymus</sup> extract in CFA and given a boosting injection after 3 weeks in order to demonstrate that these animals could still develop an experimental autoimmune disease.

### Skin tests

The development of delayed hypersensitivity to the antigen used for immunization was tested after 2 weeks by the intradermal injection of calf thymus, calf skeletal muscle and calf lymph node extracts prepared as for immunization except that their concentration was 50% w/v. Animals immunized with calf heart muscle in CFA were also skin tested with a 50% w/v calf heart muscle extract. In each instance 0.1 ml of saline and of the different antigen preparations were injected intracutaneously at different sites on the abdomen after the animals had been shaved. The tests were read 30 min, 1 hr and 24 hr after the injection, and the results were recorded positive if at the injection site an erythema with oedema developed with a diameter  $\geq 1$  cm.

### Detection of circulating antibodies

The indirect fluorescent antibody test (Weller & Coons, 1954) was used. Air dried unfixed sections of thymus, skeletal muscle, heart muscle and smooth muscle were cut at  $4\ \mu$  using a SLEE cryostat at  $-20^\circ\text{C}$ . Tissue specimens obtained from a young calf, a 2-week-old guinea-pig and from the experimental animals were frozen to  $-70^\circ\text{C}$  in an acetone-solid  $\text{CO}_2$  mixture immediately after the animals were killed.

Two antisera were used for the demonstration of antibody. First a polyvalent rabbit anti-guinea-pig  $\gamma$ -globulin serum obtained from Hyland (Hyland Division, Travenol Laboratories, Los Angeles) and secondly a rabbit anti-guinea-pig IgG serum prepared in our laboratory by immunization of a rabbit with the IgG fraction of normal guinea-pig serum. The IgG fraction was prepared by using a combined salt precipitation and chromatographic procedure (Fahey & Terry, 1967) and the absence of other immunoglobulins was confirmed by immunoelectrophoresis.

The protein content of both antisera was adjusted to 10 mg protein/ml with normal unbuffered saline and the pH was then raised to approximately 9 with a carbonate-bicarbonate buffer.

Fluorescein isothiocyanate isomer I (FITC I) (British Drug Houses Ltd, Poole, England) was used in a concentration of 1 mg for each 100 mg protein. The conjugation was done in the cold at  $4^\circ\text{C}$  over a period of 18 hr. The uncoupled FITC I was removed by passage through a Sephadex G-50 column. The filtrate was precipitated with two-thirds of its volume of saturated ammonium sulphate and the precipitated fraction dialysed overnight against veronal buffer, pH 7.2. The final fluorescein-protein ratio was calculated on a Zeiss P.M.Q. II spectrophotometer at a wavelength of 495 and 280  $m\mu$ . The protein-fluorescein ratio was found to be 0.5 for the conjugated antiserum to polyvalent immunoglobulin and 0.56 for the conjugated antiserum to IgG.

All the animals were bled 14–17 days after immunization and the sera stored at  $-20^\circ\text{C}$ . Undiluted serum and serum diluted 1:8 was applied to the frozen sections for 20 min. The



sections were then washed in veronal buffer (pH 7.2) for 30 min with continual gentle agitation. The fluorescein conjugated antisera were applied in a dilution 1:8 for 20 min and the sections were then given a final wash in veronal buffer, pH 7.2, for 1 hr with continual gentle agitation. The sections were mounted in 10% glycerol in saline and examined using a Gillet and Sibert microscope with a 100-W Iodine-Quartz lamp with primary filter 30/063 and ocular filter 10/285.

The indirect fluorescent antibody test was also used to detect the presence of antinuclear factor in the sera of the experimental animals, using frozen guinea-pig liver sections and the polyvalent anti-guinea-pig  $\gamma$ -globulin conjugated as described above.

#### *Passive haemagglutination test*

Fresh tanned sheep red cells were prepared according to the method described by Herbert (1967). Tanned sheep cells were sensitized with extracts of a guinea-pig thymus, skeletal muscle, heart muscle, smooth muscle, lymph node, thyroid and saline in a concentration of 0.1–0.25 mg tissue protein/ml. The sensitized sheep cells were incubated overnight at room temperature with doubling dilutions of the sera from the test and from the control animals. The test was read positive when haemagglutination was found in a titre  $\geq 1:20$ .

#### *Absorption procedures*

The specificity of the serum antibodies in the experimental animals immunized with thymus, skeletal muscle or heart muscle in CFA for thymus lymphocytes and for thymus myoid cells and skeletal muscle and heart muscle striations was studied by absorption experiments. Calf and guinea-pig tissue extracts of thymus, skeletal muscle, heart muscle, smooth muscle, thyroid, lymph node and liver were prepared as for immunization. Two parts of tissue extract and one part of undiluted serum were incubated in a water-bath at 37°C and shaken for 1 hr. The absorption of the antibody was then studied using the indirect immunofluorescence technique with the polyvalent rabbit anti-guinea-pig  $\gamma$ -globulin serum conjugated with FITC I.

#### *Other serological procedures*

To determine whether the serum antibodies were complement fixing all the sera were inactivated by heating at 56°C for 30 min. Cryostat sections of guinea-pig thymus, skeletal muscle, heart muscle and smooth muscle were tested in duplicate. In each instance one of the sections was treated with inactivated serum followed by the polyvalent rabbit antiserum against guinea-pig  $\gamma$ -globulin conjugated with FITC I (see above). The other section was treated with guinea-pig serum and one drop of fresh complement and then with rabbit antiserum against guinea-pig complement conjugated with FITC I (F-P ratio 0.417).

Cryostat sections of thymus, skeletal muscle and heart muscle tissue from all the animals were tested by the direct immunofluorescence method for the *in vivo* binding of  $\gamma$ -globulin using a polyvalent rabbit serum against guinea-pig  $\gamma$ -globulin conjugated with FITC I.

To test if the serum autoantibodies of animals immunized with skeletal muscle in CFA were directed against a muscle ribonucleoprotein, frozen guinea-pig muscle sections were incubated for 1 hr at 45°C with a bovine pancreas ribonuclease (British Drug Houses Ltd, Dorset, England) in a concentration of 5 mg/ml in phosphate buffered saline, pH 7.2, and afterwards used in the indirect immunofluorescence test as described above.

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### Histology

Specimens of guinea-pig thymus, spleen and cervical lymph nodes were fixed in Carnoy solution overnight. One tissue block of skeletal muscle, of heart muscle and of smooth muscle (stomach wall) was fixed in 10% formol saline. All tissue sections were stained with haematoxylin and eosin (H & E) and the thymus sections were stained also with methyl green pyronin, periodic acid-Schiff (PAS) and silver impregnation.

### Electromyography

Fourteen to 17 days after immunization each guinea-pig was anaesthetized with Nembutal®, 35 mg/kg body weight.

Two techniques were used to record the compound muscle action potential.

In the animals of Group A, a fine insulated silver wire (0.05 mm diameter) was used for recording. A loop of this wire was attached to the amplifier input and soldered connections checked by noting the absence of 50 cycle interference. The loop was then twisted and drawn through the exposed flexor digitorum of the animal's right front leg using a small suture needle. The end of the twisted double wire was cut, and the ends of each wire were bent to make small hooks. The resulting micro-bipolar electrode system was then drawn back into the muscle body (Fig. 1). During recording the exposed muscle was maintained at

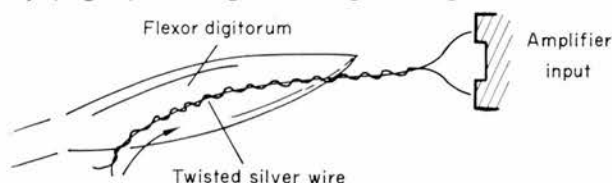


FIG. 1. Shows the silver wire micro-bipolar electrode system ready to be drawn back into the muscle body. The insulation is removed from a very small area at the end of each wire.

body temperature by covering with paraffin heated to 39°C. In animals of Group B, Copeland-Davies clip electrodes (Copeland & Davies, 1964) supplied by Electro-Physiological Instruments Ltd, Edinburgh, were used. The clips penetrated the unexposed muscle through the skin. Each clip measured 1 cm across. The two clip electrodes were placed as close together as possible. By this method recordings were made on both front limbs after they had been shaved.

In both Groups A and B the median nerve was exposed in the axilla and stimulated using silver wire electrodes (diameter 0.5 mm and shaped as small hooks) 2 mm apart. The nerve stimulation was supramaximal with a pulse duration of 100 msec. In each instance a single compound muscle action potential was observed at a sweep speed of 2 msec/cm. For tetanus a stimulation frequency of 50/sec was used and the first ten muscle action potentials observed at a sweep speed of 20 msec/cm. The first twenty-five muscle action potentials were also observed at a sweep speed of 50 msec/cm. The effect of Tensilon® on a single action potential as well as on a tetanus was tested 5–10 min after the injection of 50 µg Tensilon® intramuscularly into the right hind leg. A double pulse unit (2 GIRO, Electro-Physiological Instruments, E.P.I. Edinburgh) was used for stimulation and a recording system was designed by Electro-Physiological Instruments Ltd, Edinburgh, E.P.I. Its pre-amplifier had a gain of 1000 and was used at a time constant of 25 msec. It was incorporated in a Tele-equipment oscilloscope Model (D52). For photographic recording a Tektronix oscilloscope 502 was used with a Shackman polaroid camera (Model No. P.L.I.).

Chi-square analysis was performed to measure the significance of the incidence of thymitis in the experimental and in the control animals. Statistical evaluation of the thymus weight and the electromyographic response in the different animal groups was made using the Student *t*-test.

## RESULTS

### *Thymic histology*

Thymitis was considered to be present if there was an accumulation of small and medium

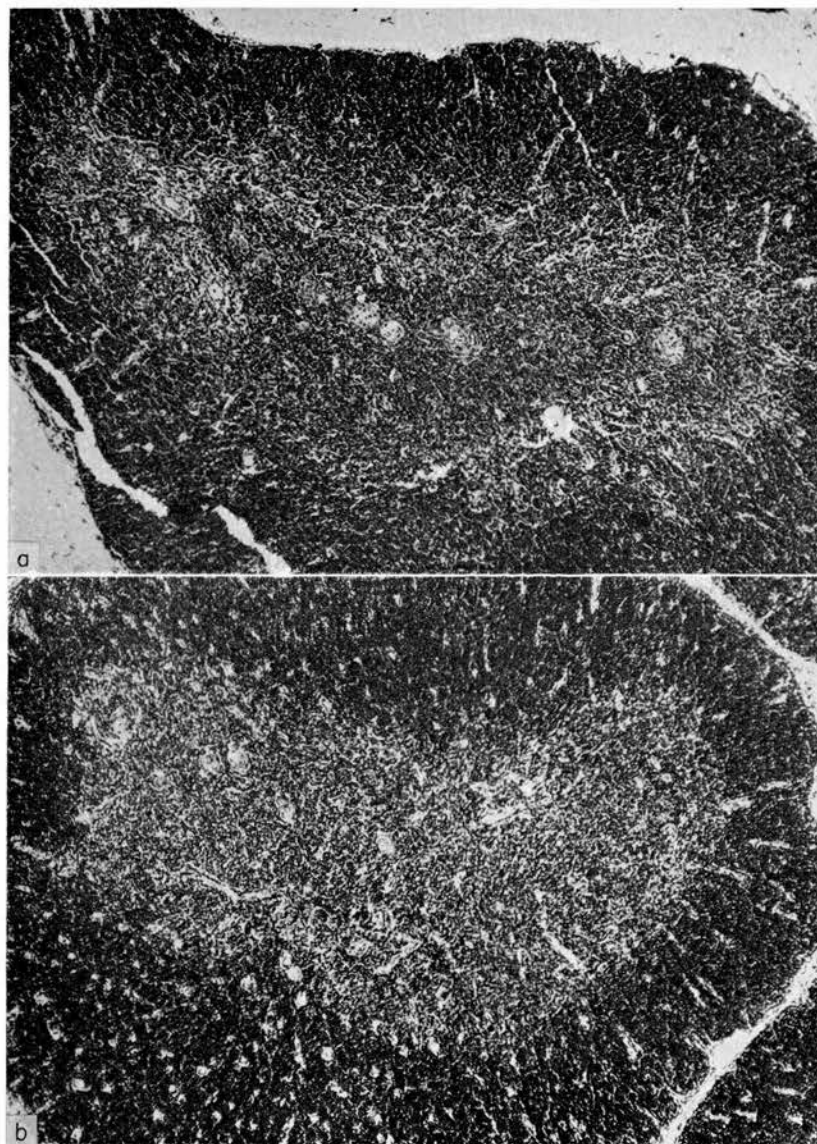


FIG. 2. Sections of guinea-pig thymus. (a) Thymus of a guinea-pig 2 weeks after immunization with calf heart muscle in CFA. Note the accumulation of lymphocytes around the Hassall's corpuscles in the thymic medulla. H & E,  $\times 70$ . (b) Normal thymus from a control animal showing an even dispersion of lymphocytes in the medulla. H & E,  $\times 70$ .

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sized lymphocytes in the thymic medulla. The accumulation of lymphocytes tended to occur around the Hassall's corpuscles (Fig. 2a). This picture was quite distinct from the histology of the normal thymus gland in the guinea-pigs in which there is a relatively even dispersion of small lymphocytes throughout the medulla of the thymus (Fig. 2b). Staining with silver impregnation showed that in some of the thymus glands there was a breakdown of the fine medullary reticulum fibre structure with the formation of a reticulum fibre barrier at the cortico-medullary border. This change was not dependent on the presence of thymitis, since it was also observed in guinea-pig thymus that was otherwise normal. There appeared to be no differences in the plasma cells, Hassall's corpuscles or cortex in the presence or absence of thymitis.

TABLE 2. Incidence of positive delayed hypersensitivity and incidence of experimental thymitis in experimental and control guinea-pigs 14 days after immunization

Thymus status	No. of animals	Antigen injected	Positive delayed hypersensitivity* (test tissue)					Incidence of thymitis	Thymus weight (mean $\pm$ SD)
			Thymus	Skeletal muscle	Heart muscle	Lymph Node	Saline		
Intact	19	Thymus	18	13	NT†	16	0	11	0.49 $\pm$ 0.25
	18	Skeletal muscle	16	13	NT	11	0	9	0.56 $\pm$ 0.24
	9	Heart muscle	9	8	9	8	0	2	0.51 $\pm$ 0.16
	9	Lymph node	7	6	NT	8	0	1	0.55 $\pm$ 0.16
	8	Saline	0	0	NT	0	0	1	0.47 $\pm$ 0.11
	10	—	0	0	NT	0	0	0	0.5 $\pm$ 0.22
Thymectomized	8	Thymus	6	4	NT	6	0	—	—
	7	Skeletal muscle	7	6	NT	7	0	—	—
	4	Lymph node	4	1	NT	4	0	—	—

\* No. of animals with positive skin reactions after 24 hr.

† NT, Not tested.

The incidence of thymitis (Table 2) recorded in animals injected with calf thymus in CFA (eleven out of nineteen) and in those immunized with calf skeletal muscle in CFA (nine out of eighteen) is significantly higher than the incidence in control animals (two out of twenty-two) with  $P$  values of  $<0.001$  and  $<0.01$ , respectively. The occurrence of thymitis in two out of nine animals immunized with heart muscle in CFA was not significant when compared with that in the control animals ( $P>0.5$ ). Likewise there was no statistical difference in the incidence of thymitis in animals injected with lymph node in CFA (one out of nine) or in animals injected with saline in CFA (one out of eight) compared to untreated guinea-pigs (none out of ten).

*Thymus weight*

The weight of the thymus in the different animal groups is given in Table 2. No significant differences were observed between the groups ( $P>0.2$ ).

*Histology of other tissues*

No abnormalities were noted in sections of spleen, lymph node and smooth muscle either in the experimental or in the control animals. In one guinea-pig immunized with thymus an inflammatory reaction was found in a muscle section taken from the proximal part of the



right hind leg. In one animal immunized with skeletal muscle an inflammatory focus in the myocardium was recorded. A small perivascular focus of inflammation was also observed in the myocardium in two of the guinea-pigs injected with heart muscle in CFA. No abnormalities in skeletal muscle or myocardium were noted in the remaining test or control animals. All guinea-pigs immunized with a crude thyroid extract in CFA after thymectomy developed histological evidence of mild to severe thyroiditis.

#### *Delayed hypersensitivity*

The number of animals giving a positive skin reaction for delayed hypersensitivity after 24 hr is shown in Table 2. No tissue specific reaction was obtained. In about 30% of the immunized animals the skin reaction showed a central necrosis after 24 hr. No immediate type of reaction was seen in any of the animals at 30 min and 1 hr after the injection. None of the animals injected with saline and none of the untreated guinea-pigs gave a positive reaction to any of the test tissues. No difference was recorded in the development of delayed hypersensitivity in guinea-pigs when the thymus was intact or after thymectomy (Table 2).

#### *Circulating antibodies*

The antibodies detected in the serum 14 days after immunization with the various tissues is given in Table 3. Results obtained in the indirect immunofluorescence test using heterologous (calf) homologous (2-week-old guinea-pigs) and autologous tissue sections were similar.

TABLE 3. Serological studies in experimental and control guinea-pigs 13 days after immunization

Thymus status	No. of animals	Antigen injected	Indirect immunofluorescence test*				ANF†
			Thymus		Skeletal muscle (striations)	Heart muscle (striations)	
			Cytoplasm of lymphocytes	Myoid cells			
Intact	19	Thymus	17	0	0	0	5
	18	Skeletal muscle	0	17	17	17	6
	9	Heart muscle	0	3	5	5	5
	9	Lymph node	(8)	0	0	0	3
	8	Saline	0	0	0	0	0
	10	—	0	0	0	0	0
Thymectomized	8	Thymus	7	0	0	0	(2)
	7	Skeletal muscle	0	7	7	7	(1)
	4	Lymph node	(4)	0	0	0	0

Numbers in parentheses indicate that the reactions are only weakly positive.

\* Using autologous tissue sections and serum dilutions 1:8.

† Using homologous guinea-pig liver sections and undiluted serum in the indirect fluorescence test.

In about 10% of the control animals the undiluted sera gave weak but definite fluorescence reactions with the striations of skeletal muscle and heart muscle and the cytoplasm of lymphocytes and myoid cells of the thymus. When the test sera were diluted 1:8 no positive staining was found with the sera of the control animals. No significant difference ( $P > 0.5$ )

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was recorded in the incidence of circulating antibodies in thymectomized and intact animals.

Out of twenty-seven guinea-pigs injected with thymus twenty-five gave a positive fluorescence reaction with the cytoplasm of the thymus lymphocytes (Fig. 3). No reaction was recorded with skeletal muscle or with heart muscle sections. A similar but weak reaction was found in seven out of nine guinea-pigs injected with lymph node. Twenty-four out of

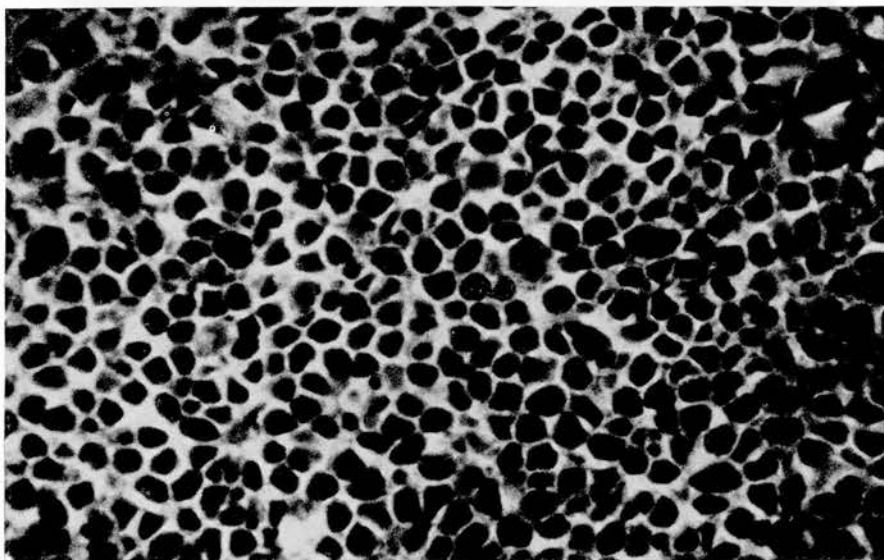


FIG. 3. Indirect immunofluorescence test using a cryostat section of guinea-pig thymus and the serum of a guinea-pig 2 weeks after immunization with calf thymus in CFA. There is a positive reaction with the cytoplasm of the thymus lymphocytes.  $\times 320$ .

twenty-five animals immunized with skeletal muscle and five out of nine animals with heart muscle showed positive fluorescence reactions with the cytoplasm of the thymus myoid cells (Fig. 4a), and with the striations of skeletal muscle (Fig. 4b) and heart muscle (Fig. 4c). A few sera also showed positive fluorescence reactions with Hassall's corpuscles and weak staining of the connective tissue septa in the thymus (Fig. 4a). None of the sera reacted with smooth muscle. Identical fluorescence reactions were obtained using conjugates prepared from a rabbit anti-guinea-pig polyvalent  $\gamma$ -globulin serum and from a rabbit anti-guinea-pig IgG serum.

Table 4 shows the distribution of the antibody titres in the test and control animals. In general the results compare favourably with those obtained by the indirect immunofluorescence test. In animals immunized with thymus in CFA the tanned red cell titre ranged up to 1:1280, and in animals sensitized with skeletal muscle in CFA the titres ranged up to 1:2560 (Fig. 5). The results demonstrate the tissue specificity of the serum antibodies raised in the experimental animals injected with different tissue homogenates, since guinea-pigs immunized with thymus in CFA reacted only with thymus or lymph node sensitized red cells and not with erythrocytes sensitized with skeletal muscle, heart muscle, smooth muscle or thyroid extracts nor with the saline control. A similar but weaker reaction pattern was found in guinea-pigs immunized with lymph node in CFA. Animals injected with skeletal muscle or heart muscle in CFA reacted only with these tissues, with the exception of three

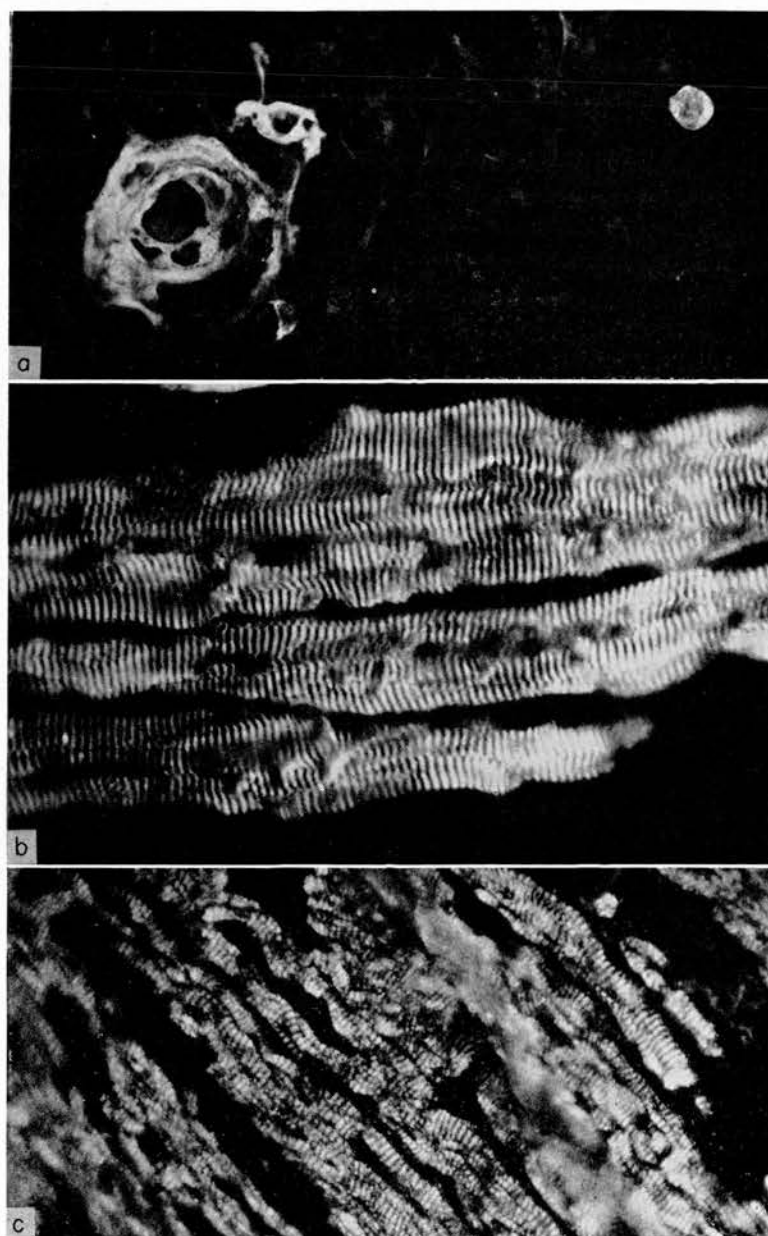


FIG. 4. Indirect immunofluorescence tests using sections of guinea-pig thymus, skeletal muscle and heart muscle and the serum from a guinea-pig 2 weeks after immunization with calf skeletal muscle in CFA. (a) Positive reaction with the cytoplasm of a thymus myoid cell and with a Hassall's corpuscle. There is also weak reaction with the connective tissue septa.  $\times 280$ . (b) A positive reaction with the cross striations of skeletal muscle.  $\times 630$ . (c) A positive reaction with the cross striations of heart muscle.  $\times 630$ .

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TABLE 4. Serum antibody titre of experimental and control guinea-pigs obtained by the passive haemagglutination test after 14 days immunization

Thymus status	No. of animals	Antigen injected	Test tissue											
			Thymus			Skeletal muscle			Heart muscle			Smooth muscle		
			1:20	1:40	$\geq 1:80$	1:20	1:40	$\geq 1:80$	1:20	1:40	$\geq 1:80$	1:20	$\geq 1:80$	$\geq 1:20$
Intact	19	Thymus	5*	4	6	—	—	—	—	—	—	—	—	—
	18	Skeletal muscle	3	—	—	3	3	8	4	7	—	3	—	—
	9	Heart muscle	—	—	—	4	3	—	4	3	—	—	—	—
	9	Lymph node	4	—	—	—	—	—	—	—	—	—	—	—
	8	Saline	—	—	—	—	—	—	—	—	—	—	—	—
Thymectomized	10	—	—	—	—	—	—	—	—	—	—	—	—	—
	8	Thymus	3	2	—	—	—	—	—	—	—	—	—	—
	7	Skeletal muscle	1	—	—	2	1	3	2	1	3	1	—	—
	4	Lymph node	1	1	—	—	—	—	—	—	—	—	1	—

\* No. of animals showing antibody titre at the serum dilutions indicated.



guinea-pigs which in addition showed a weak reaction with thymus sensitized cells. This latter finding is explained by the presence of a common antigen in skeletal muscle, heart muscle and thymus.

The serum antibodies in guinea-pigs immunized with thymus or lymph nodes could be selectively absorbed with thymus or lymph node extracts (calf or guinea-pig) prepared as for immunization. Similar extracts of calf and guinea-pig skeletal muscle, heart muscle, smooth muscle, thyroid and liver were ineffective in this respect. Antibodies in the serum of

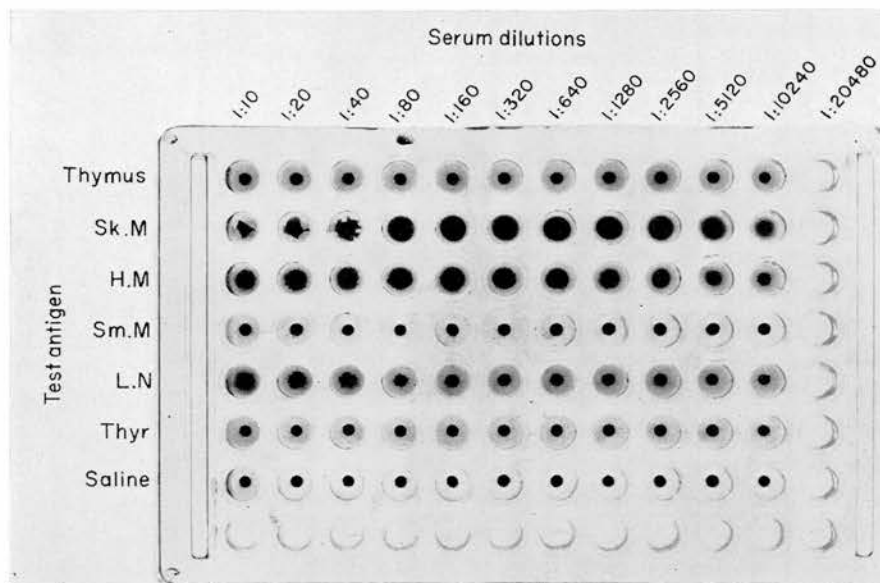


FIG. 5. Passive haemagglutination tests demonstrating the reaction pattern of serum antibodies from a guinea-pig 2 weeks after immunization with calf skeletal muscle in CFA. Agglutination occurred with sheep red cells sensitized with skeletal muscle (Sk.M) or with heart muscle (H.M) but not with cells sensitized with thymus, smooth muscle (Sm.M), lymph node (L.N) or thyroid (Thyr) extracts.

guinea-pigs immunized with skeletal muscle were readily absorbed by an extract of skeletal muscle but only weakly by an extract of heart muscle. Antibodies in the sera of animals immunized with heart muscle were readily absorbed by either skeletal muscle or heart muscle.

The detection of the antibodies by the indirect immunofluorescence test was not dependent on complement. Inactivation of the sera did not affect the results. Consistently negative results were obtained when positive sera by the indirect immunofluorescence method were inactivated, applied to the appropriate tissue sections, the sections then treated with fresh guinea-pig complement and counterstained with a rabbit anti-guinea-pig complement conjugate.

Definite positive tests for antinuclear factor were found in seven out of nine guinea-pigs immunized with thymus in CFA, six out of eighteen animals immunized with heart muscle and skeletal muscle in CFA and three out of nine guinea-pigs immunized with lymph node in CFA. Weak reactions for ANF were observed in three out of five guinea-pigs thymectomized before immunization with thymus or skeletal muscle (Table 3). No correlation was found between the incidence of thymitis and the presence of ANF in the serum.

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Direct immunofluorescence tests for guinea-pig  $\gamma$ -globulin in thymus, skeletal muscle and heart muscle sections gave negative results in both the experimental and in the control animals.

Pretreatment of skeletal muscle, heart muscle and thymus sections with a ribonuclease prevented positive immunofluorescence reactions with skeletal muscle and heart muscle striations as well as with thymus myoid cell cytoplasm, but it did not prevent positive staining of the Hassall's corpuscles and connective tissue septa in the thymus.

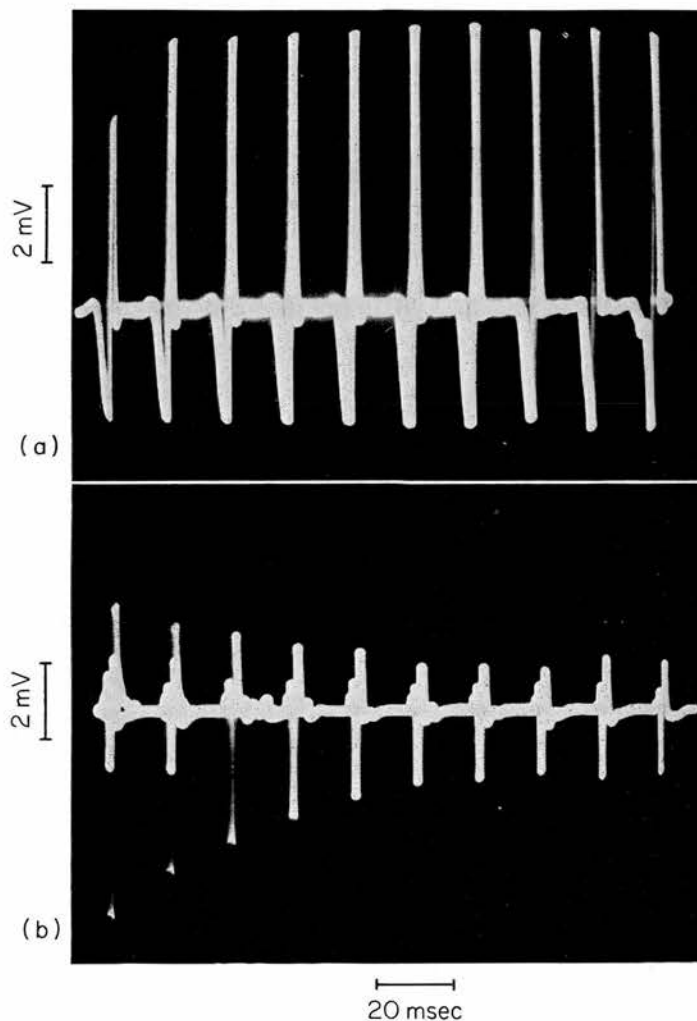


FIG. 6. Record of the muscle action potentials in the flexor digitorum following supramaximal nerve stimulation at a rate of 50/sec using Copeland-Davies clip electrodes. (a) Normal response in which the height of the successive muscle responses is maintained. The height of the first action potential as shown is foreshortened by the photographic tube. (b) Decreasing response in an animal immunized with calf heart muscle in CFA and in which thymitis was present. The tenth action potential is 36.6% of the first.

*Neuromuscular transmission*

The behaviour of the first ten muscle action potentials during tetanic supramaximal nerve stimulation was used to study neuromuscular transmission (Harvey & Masland, 1941). The ratio of the amplitude of the tenth to the first action potential was noted. In the control animals the lower confidence limit for this ratio was 0.81. The chance that the ratio would be

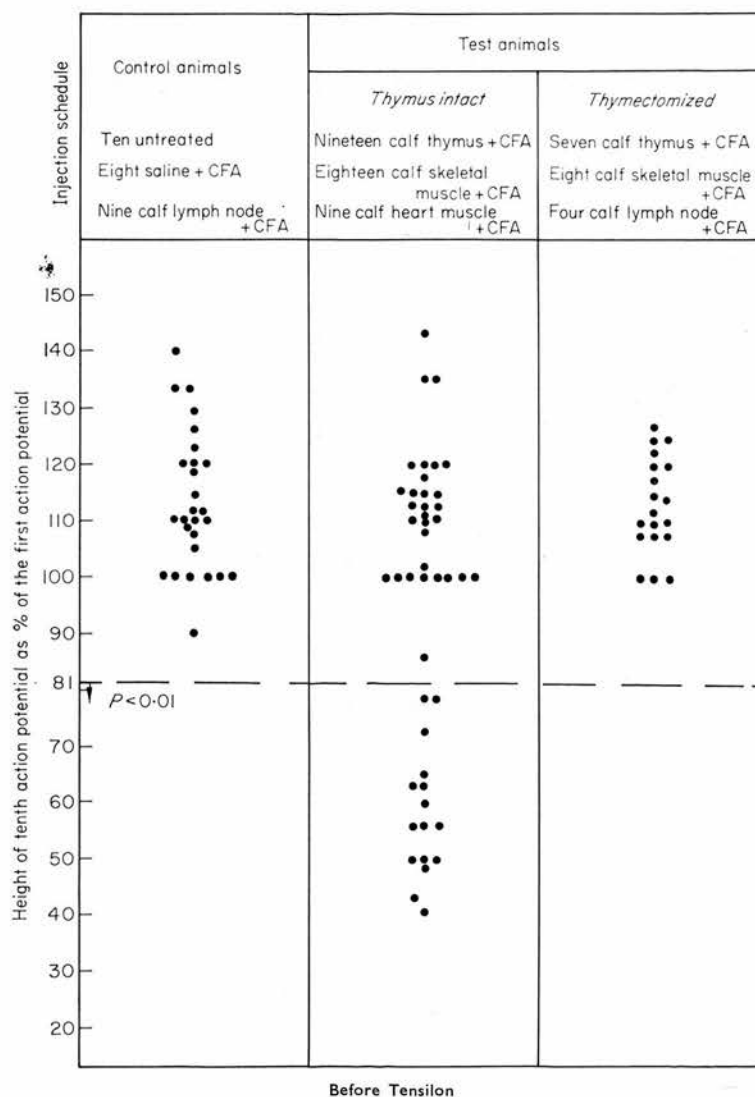


FIG. 7. The electromyographic response to supramaximal nerve stimulation at a rate of 50/sec in animals of Groups A and B combined before the injection of Tensilon. In animals of Group B, the individual results shown are the mean of the recordings in each front limb. From the findings in control animals, a decline in the height of the tenth action potential to less than 81% of the first was unlikely ( $P < 0.01$ ). Seven of the thymus-immunized, seven of the skeletal muscle-immunized and two of the heart muscle-immunized animals had a significant decline in the muscle action potentials compared to the tetanus pattern observed in the control animals.

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smaller than this is unlikely ( $P < 0.01$ ). Therefore, guinea-pigs showing a ratio of less than 0.81 were regarded as having abnormal neuromuscular transmission (Fig. 6a and b).

In the animals of Group A, three out of ten guinea-pigs immunized with thymus in CFA and four out of ten animals immunized with skeletal muscle in CFA showed a decline in the successive muscle responses over the first ten stimuli to  $63 \pm 23.4\%$  (mean  $\pm$  SD) and to

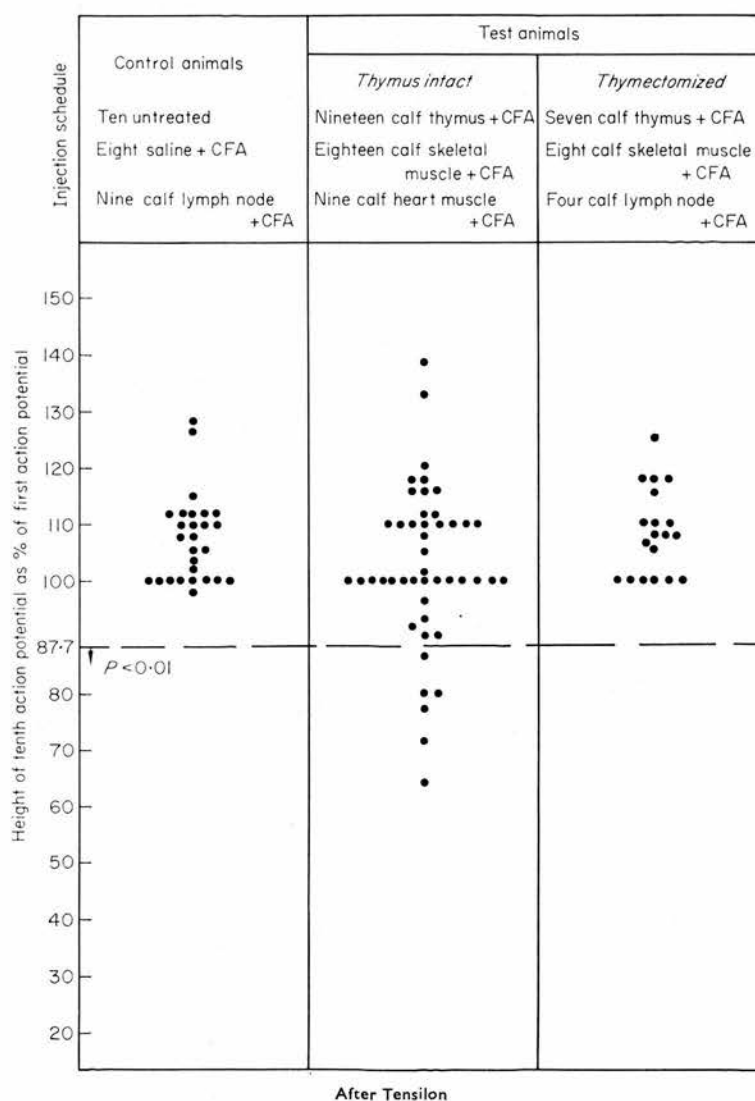


FIG. 8. The same as Fig. 7 but 10 min after 50  $\mu$ g Tensilon intramuscularly. The lower confidence limit in the control animals was 87%. Note, in comparison to Fig. 7, the improvement in the tetanus muscle reaction in all animals with a partial neuromuscular block. In six guinea-pigs, however, the tetanus pattern was still subnormal.

$59.3 \pm 6.0\%$  of the initial action potentials, respectively. In contrast, in the control animals of this group an increase in the action potentials to  $118.3 \pm 36.9\%$  was observed (Fig. 7).



In those animals with a partial neuromuscular block a significant improvement in their successive muscle action potentials was observed 5–10 min after the intramuscular injection of 50  $\mu$ g Tensilon ( $P < 0.05$  for thymus-immunized, and  $P < 0.02$  for skeletal muscle-immunized animals, respectively). The tetanus pattern, however, remained subnormal. No marked affect was found in the tetanus pattern of control animals after Tensilon (Fig. 8).

In the animals of Group B, two out of nine guinea-pigs immunized with heart muscle in CFA showed a decline to  $60.6 \pm 10.0\%$  (mean  $\pm$  SD) in the right and to  $71.4 \pm 2.2\%$  in the

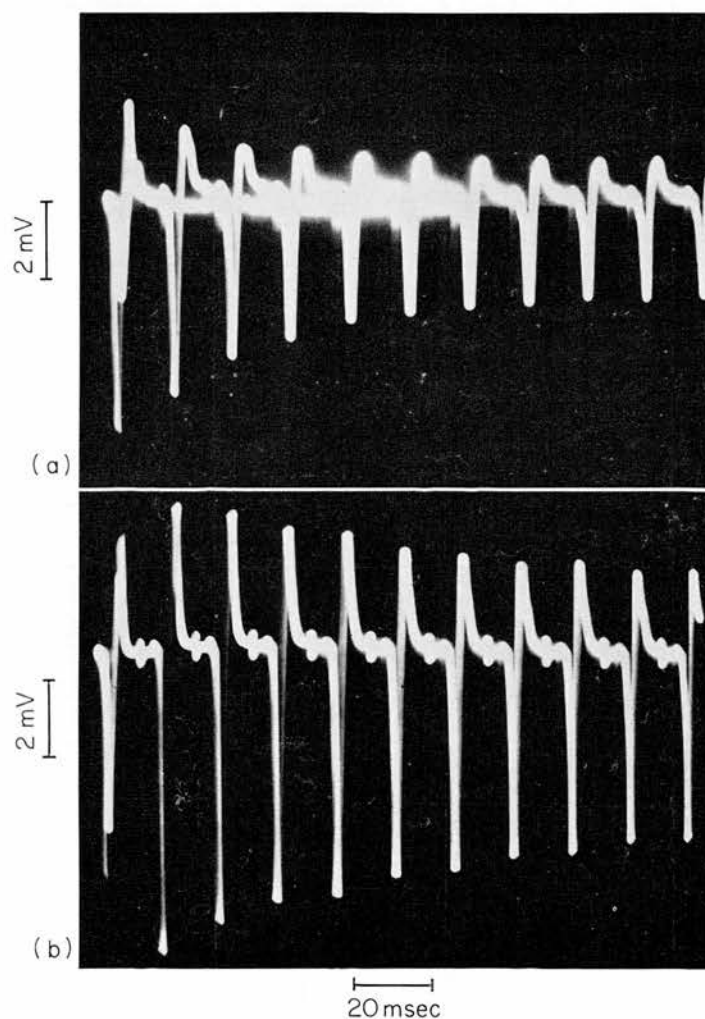


FIG. 9. Muscle action potentials in the flexor digitorum following supramaximal nerve stimulation at a rate of 50/sec. The recordings were made before and after the injection of Tensilon in a guinea-pig with thymitis 2 weeks after immunization with calf thymus in CFA. (a) Before the injection of Tensilon the height of the tenth action potential is 32% of the first. (b) Ten minutes after the injection of Tensilon the decremental response is markedly improved, the tenth muscle action potential being 65% of the first. The height of the first action potential as shown is foreshortened by the photographic tube.

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left front limb. Three out of eight animals immunized with skeletal muscle in CFA showed a decline in their muscle action potentials over the first ten stimuli to  $64 \pm 28.1\%$  in the right and to  $51 \pm 11.7\%$  in the left front limb. In four out of nine guinea-pigs immunized with thymus in CFA, a decline was found to  $62.8 \pm 29.6\%$  in the right and to  $66.9 \pm 10.7\%$  in the left front limb. In one of the animals immunized with skeletal muscle, a marked

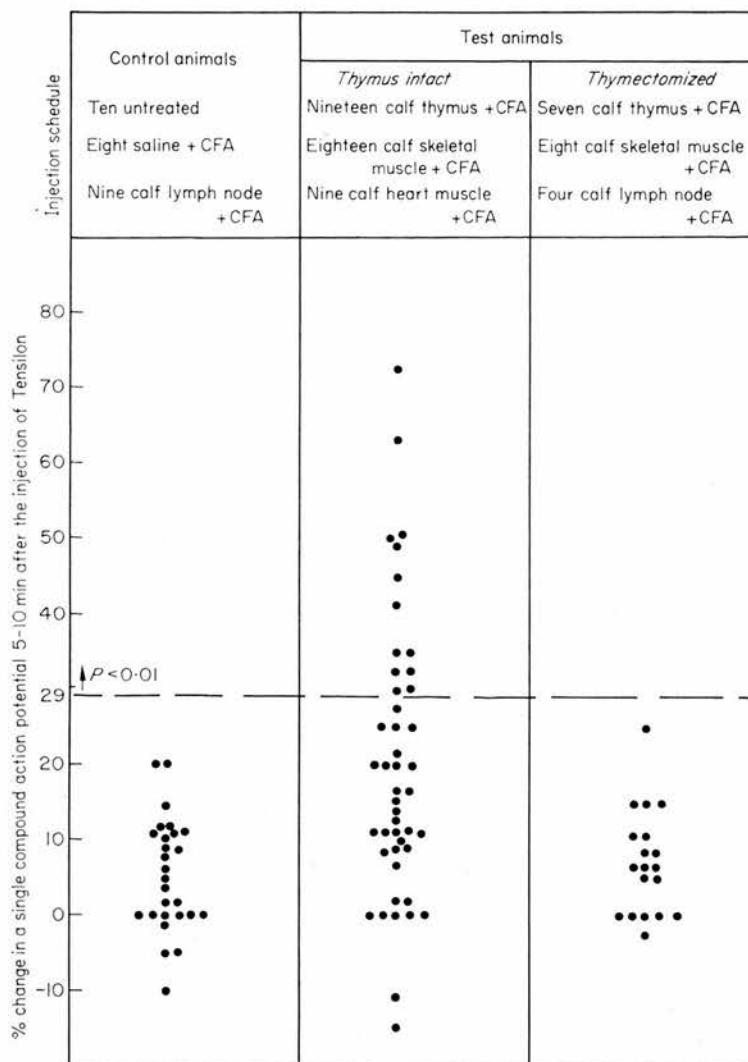


FIG. 10. The change in a single compound muscle action potential after the injection of Tensilon in animals of Groups A and B. In guinea-pigs of Group B, the results for the individual animals are shown as the mean of the recordings in the right and left front limbs. From the results obtained in the control animals a rise in a single compound action potential after Tensilon of more than 29% was unlikely ( $P < 0.01$ ). The action potentials of six guinea-pigs immunized with thymus, six immunized with skeletal muscle and one immunized with heart muscle in CFA showed a significant increase after the injection of Tensilon.

difference in the muscle tetanus pattern was found between the front limbs with a decline in the successive muscle responses to 78.9% in the right and to 40% in the left front limb. In the control guinea-pigs of this group an increase in the successive muscle responses to  $111.3 \pm 23.5\%$  in the right and to  $110.4 \pm 25.6\%$  in the left front leg was recorded. A similar increase to  $113.1 \pm 17.5\%$  in the right and to  $110.4 \pm 20.1\%$  in the left front limb was found in those animals which were thymectomized and 1 week later immunized with either thymus or skeletal muscle in CFA. Again after the injection of 50  $\mu$ g Tensilon® a significant improvement in the muscle tetanus pattern was observed in animals showing a partial neuromuscular block ( $P < 0.05$ ,  $P < 0.02$  and  $P < 0.01$  for animals immunized with heart muscle, skeletal muscle and thymus, respectively), but the tetanus pattern did not revert to a completely normal picture in all animals (Fig. 9a and b). In the control animals of this group no effect was seen (Fig. 8) after the injection of Tensilon.

In those animals of Group A in which a significant decline in the successive muscle responses was observed, the height of a single muscle action potential following a single nerve stimulus was  $3.0 \pm 0.57$  mV (mean  $\pm$  SD). This was not significantly different from the mean height of a single action potential in the control animals of this group,  $3.6 \pm 0.48$  mV ( $P > 0.1$ ). The mean height of a single action potential in guinea-pigs of Group B which had shown a partial neuromuscular block during tetanic nerve stimulation was  $7.17 \pm 2.84$  mV in the right and  $7.14 \pm 3.85$  mV in the left front limb. This was significantly different ( $P < 0.01$ ) from the single muscle responses of  $9.59 \pm 2.4$  mV in the right and  $10.85 \pm 6.24$  mV in the left front leg of the control animals and of the guinea-pigs thymectomized prior to immunization. The height of a single action potential in animals thymectomized prior to immunization (with  $12.44 \pm 6.02$  mV right and  $11.73 \pm 4.4$  mV six out of eight) was not significantly different ( $P > 0.1$ ) from that recorded in the control animals of this group.

From the study in the control animals, it could be said that a rise in a single muscle action potential after the intramuscular injection of 50  $\mu$ g Tensilon by more than 29% would be unlikely ( $P < 0.01$ ). However, out of the forty-six animals in Groups A and B, thirteen guinea-pigs showed an increase in a single muscle action potential after the injection of Tensilon beyond 29% with a range between 29 and 73%. All these animals had evidence of a partial neuromuscular block during tetanic supramaximal nerve stimulation. In one guinea-pig immunized with thymus in CFA, one immunized with skeletal muscle in CFA and in one immunized with heart muscle in CFA with a significant decline in the successive muscle responses during tetanic supramaximal nerve stimulation, an increase in a single action potential of only 20–25% was recorded after the injection of Tensilon (Fig. 10).

## DISCUSSION

In contrast to the negative findings published by Strauss (1963) and by Parkes (1966), who were unable to produce a model of myasthenia gravis in experimental animals, the present paper describes the production of thymic abnormalities and of partial neuromuscular block in guinea-pigs by immunization with either calf thymus or calf skeletal muscle extract emulsified in CFA. Our findings are similar to those reported by Goldstein & Whittingham (1966, 1967).

The thymic abnormalities consisted of an accumulation of small and medium sized lymphocytes around the Hassall's corpuscles in the thymic medulla. This is referred to as a thymitis. Germinal centres were not seen. A distortion of the fine reticulum fibre structure in the

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thymic medulla, with the formation of a reticulum fibre barrier at the cortico-medullary border, did not always correspond with the presence of thymitis as described by Goldstein & Whittingham (1967). Our own unpublished experiments as well as recent results obtained by Goldstein & Hofmann (1968) and Goldstein, Strauss & Pickeral (1969) show that the incidence of thymitis may be increased by immunization of rats or guinea-pigs with homologous thymus or skeletal muscle tissue in CFA.

Experimental thymitis was also produced in two out of nine animals immunized with calf heart muscle in CFA which was, however, statistically not significantly different from the control animals. Both these animals showed evidence of partial neuromuscular block. The potency of heart muscle to initiate an experimental thymitis is probably due to a common antigen shared by skeletal muscle, heart muscle and thymus myoid cells (Van der Geld & Strauss, 1966; Beutner *et al.*, 1962). The low incidence of thymitis using heart muscle as antigen may be explained by a smaller content of thymus specific antigens in heart muscle tissue. In a careful study by Goldstein *et al.* (1969) on the capacity of thymus and skeletal muscle tissue to induce experimental thymitis, thymic tissue was found to be much more antigenic than skeletal muscle tissue, probably because of a smaller content of thymus specific antigen in the muscle tissue.

Lymphorrhages in skeletal muscle and/or myocarditis have been described in about 23% of myasthenia gravis patients (Fenichel, 1966; Mendelow & Jenkins, 1954). Comparable histological changes were only observed in one out of twenty-seven guinea-pigs immunized with thymus in CFA which showed a lymphocytic infiltration in a muscle section from the upper hind limb. In one out of twenty-five guinea-pigs injected with skeletal muscle in CFA a local lymphocytic infiltration with necrosis of the muscle tissue was seen in the myocardium. Both these animals showed the features of experimental thymitis and a partial neuromuscular block. In two out of nine animals immunized with heart muscle in CFA a local lymphocytic infiltration in the myocardium was observed. The low incidence of myocardial lesions in our experiments compared to that described by Davies *et al.* (1964) is probably due to the different methods used for antigen preparation and to the different immunization schedules. Furthermore, a higher incidence of myositis in skeletal muscle may have been achieved if tissue had been examined from a larger selection of sites. None of the control animals showed lymphorrhages in skeletal muscle or myocarditis.

We were not able to correlate the development of thymitis with any pattern of delayed hypersensitivity or circulating antibody response. Guinea-pigs immunized with thymus or lymph node in CFA developed serum autoantibodies reacting with the thymus lymphoid cells but not with the myoid cells or with skeletal muscle or heart muscle probably because of insufficient myoid antigen in the calf thymus extract used for immunization (Van der Geld & Strauss, 1966). Guinea-pigs immunized with skeletal muscle or heart muscle in CFA developed antibodies against the striations of skeletal muscle, cross-reacting with heart muscle striations and thymus myoid cells. In this respect, the antibodies raised in the experimental animals had the same reactivity as serum antibodies found in patients with myasthenia gravis. However, in patients with myasthenia, there are two types of antibody reacting with muscle antigen; one of them is complement fixing and reacts with skeletal muscle only and the other is not complement fixing and reacts with both skeletal muscle and heart muscle (Van der Geld & Strauss, 1966; Beutner *et al.*, 1962). Complement fixing antibodies reactive with muscle antigens could not be produced in the guinea-pig.

A rapid decline in successive muscle responses to supramaximal nerve stimulation and



reversion to a normal tetanus pattern in response to an anticholine-esterase drug is characteristic of myasthenia gravis (Harvey & Masland, 1941; Desmedt, 1966). We have confirmed results reported by Goldstein & Whittingham (1966) that a similar pattern of a neuromuscular block can be produced in experimental animals by immunological techniques. More recently Goldstein & Hofmann (1968) have been able to demonstrate in rats with experimental thymitis a decrease in the miniature muscle end-plate potentials which is another characteristic feature of myasthenia gravis in man (Hofmann & Stemmer, 1963; Thesleff, 1966).

In contrast to bipolar needle electrodes which are frequently used for electromyographic recordings, we employed either Copeland-Davies clip electrodes or a fine twisted silver wire drawn through the muscle as recording systems. Both these techniques were found to be superior to bipolar needle electrodes for electromyography in guinea-pigs in terms of stability in the recording of a constant number of muscle fibre action potentials during the test procedure. Using Copeland-Davies clip electrodes a larger number of contracting units were recorded than by the fine silver wire method. Both these methods, however, have the same limitation as the bipolar needle electrodes in so far that they do not record the same constant number of contracting units in different animals. This fact may explain why the single action potential following supramaximal nerve stimulation in animals with experimental thymitis was not always significantly lower than the action potentials recorded in the control animals. Thus, when these types of electrodes are used, the recording of the muscle tetanus pattern during supramaximal nerve stimulation is the better parameter for detecting partial neuromuscular block.

The fact that in some of the animals Tensilon did not significantly improve the height of single compound action potentials and that in some animals with a partial neuromuscular block Tensilon did not restore the tetanic muscle reaction to normal may have been due to insufficient dosage of Tensilon, particularly as this was given intramuscularly. On the other hand, in patients with myasthenia gravis Tensilon does not invariably restore neuromuscular transmission to normal (Osserman & Genkins, 1966).

The occurrence of a marked difference in the muscle tetanus pattern in the front limbs of one guinea-pig immunized with skeletal muscle in CFA suggests that the degree of neuromuscular block produced in experimental animals may not be the same throughout the whole of the skeletal musculature.

The presence of thymitis in all animals with a partial neuromuscular block and the observation that a neuromuscular block could not be induced in thymectomized animals by immunization procedures indicate that the thymus gland is important in the production of experimental myasthenia gravis. Furthermore, in patients with myasthenia gravis of short duration thymectomy may be beneficial. Thymectomy in animals with thymitis and partial neuromuscular block has been shown to restore neuromuscular transmission to normal within 3-4 days in contrast to hemi-thymectomy or sham-thymectomy in such animals when it takes 7-10 days for the neuromuscular transmission to come back to normal (Kalden, Williamson & Irvine, 1969 in preparation). These observations support the hypothesis that an autoimmune mechanism may be important in at least some cases of myasthenia gravis. Strauss *et al.* (1966) and Goldstein (1966) have suggested that the thymus may be a source of a factor which has normally a regulative influence on neuromuscular transmission but which blocks neuromuscular transmission when secreted in excess, as may happen in the presence of thymitis. However, attempts to extract from the thymus a substance with such

*Experimental thymitis*

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properties have so far produced only equivocal results (Wilson, Obrist & Wilson, 1953; Rider, 1955; Parkes & McKinna, 1967; Goldstein, 1968).

## ACKNOWLEDGMENTS

We wish to thank Miss Sylvia Wheeler for preparing the histological sections. We are grateful to Mr W. T. S. Austin (Chief Technician, Physiology Department, Edinburgh University) for his help in performing the electromyographical studies and Mr J. Lowe for housing and assisting with the test animals.

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EXPERIMENTAL MYASTHENIA GRAVIS

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\*Lancet, (1969) ii, 638-639



*Letter to the Editor**reprinted from THE LANCET, September 20, 1969, pp. 638-639***EXPERIMENTAL MYASTHENIA GRAVIS**

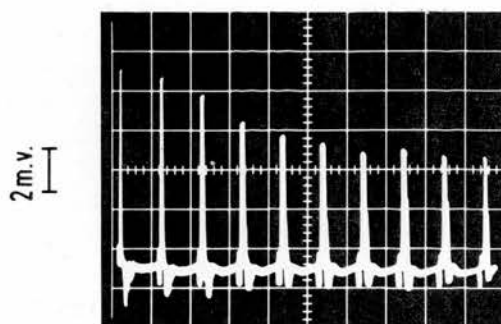
SIR,—Dr. Vettters and his colleagues (July 5, p. 28) report that they are unable to confirm the findings of others<sup>1-6</sup> that experimental myasthenia gravis can be produced by immunological techniques. These workers might, perhaps, have referred to two important positive contributions<sup>4,5</sup> published at the end of 1968 and at the beginning of 1969. In the first of these Goldstein and Hofman<sup>4</sup> demonstrated a significant decrease in the miniature end-plate potentials, in association with thymitis, in rats immunised with homologous-thymus extract in complete Freund's adjuvant (C.F.A.). In the second paper, Goldstein et al.<sup>5</sup> showed that muscle tissue was effective in producing thymitis and neuromuscular block in doses down to 50 µg. while thymus was effective in amounts as little as 5 µg.

The production of thymitis is an essential step in producing partial neuromuscular block.<sup>1,6</sup> It appears that Dr. Vettters and his colleagues have not succeeded in producing significant changes in the thymus, although their immunisation procedure was similar to that used by others.<sup>1-3,6</sup> They do not mention whether their immunised animals produced circulating antibodies or showed delayed hypersensitivity to thymus or skeletal-muscle antigen. Immunisation experiments are difficult to standardise and it is hazardous to read too much into negative results from one batch of animals.

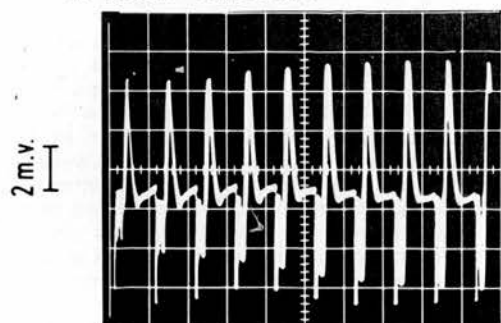
1. Goldstein, G., Whittingham, S. *Lancet*, 1966, ii, 315.
2. Goldstein, G., Whittingham, S. *Clin. exp. Immunol.* 1967, 2, 257.
3. Oppenheim, T. J., Goldstein, G. *Nature, Lond.* 1969, 222, 192.
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6. Kalden, J. R., Williamson, W. G., Johnston, R. J., Irvine, W. J. *ibid.* 1969, 5, 321.

2

## A. Nerve Stimulation



## B. Muscle Stimulation



20m.ses

Fig. 1—Electromyographic response of rat forearm muscle to indirect (A) and direct (B) supramaximal stimulation at a rate of 50 per second.

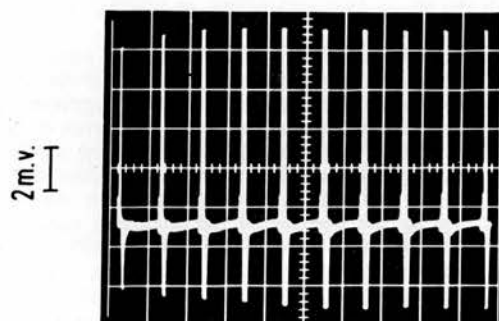
The recording was made two weeks after immunisation with homologous thymus in C.F.A.

Dr. Vettors and his colleagues raise a number of points about electromyographic techniques. They found that bipolar needle electrodes were not suitable for electromyographic muscle recordings. We use either Copeland-Davies clip electrodes<sup>7</sup> or a fine twisted silver wire.<sup>8</sup> The comment that the recording of the first 10 muscle responses to a fast (fifty-per-second) train of stimuli is "not suitable

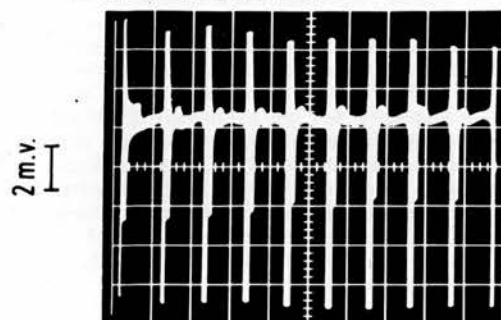
7. Copeland, J. C., Davies, C. T. M. *Lancet*, 1964, i, 416.

3

## A. Nerve Stimulation



## B. Muscle Stimulation



—|—  
20m.ses

**Fig. 2—Electromyographic response of rat forearm muscle to indirect (A) and direct (B) supramaximal stimulation at a rate of 50 per second in a control animal.**

for comparable measurements unless it can be guaranteed that the muscle contraction is isometric," has been checked in the following way. A recording of the first 25 muscle responses to supramaximal nerve stimulation (routinely done in all our experiments) gives the same results as the first 10 successive muscle responses. In animals showing a rapid decline in the first 10 muscle action potentials during tetanic nerve stimulation, no significant increase was observed thereafter. The same results were obtained as regards the presence or absence of neuro-

muscular block, whether the front limbs were fixed by pins through the elbow joint and wrists, or whether the wrists alone were fixed.

In the experiment illustrated in fig. 1, we studied the first 10 successive muscle responses to supramaximal nerve stimulation, and subsequently to supramaximal direct muscle stimulation, in the flexor digitorum of both front limbs. For stimulation and for recording a pair of Copeland-Davies clip electrodes was used (diameter of clip, 0.5 cm.). Indirect and direct stimulation was done without altering the position of the recording electrodes. We used female inbred Wistar rats of about 200 g. body-weight, immunised with homologous thymus extract in C.F.A. (11 animals), or with saline in C.F.A. (10 animals). With indirect stimulation, 5 of the thymus-immunised rats showed a significant ( $p < 0.01$ ) decline in the first 10 successive muscle responses, but a normal muscle tetanic pattern when the flexor digitorum was stimulated directly. All the animals with a partial neuromuscular block showed thymitis on histological examination, as previously described.<sup>2,5,6</sup> There was no significant decline in the first 10 muscle responses, nor any significant difference in the muscle-reaction pattern to direct or indirect stimulation in the control animals (fig. 2). These findings, which indicate that a block in neuromuscular transmission may indeed be produced in thymus-immunised rats, are in keeping with the more precise experiments of Goldstein and Hofman.<sup>4</sup> But, if no thymitis is produced, there will be no neuromuscular block.

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THE EFFECT OF THYMECTOMY, HEMITHYMECTOMY AND  
SHAM THYMECTOMY ON EXPERIMENTAL MYASTHENIA GRAVIS  
IN GUINEA-PIGS

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CLIN. EXPER. IMMUNOL. (1970) 6, 519-530

### SUMMARY

Experimental thymitis and a partial neuromuscular block were produced in guinea-pigs by immunisation with homologous thymus or skeletal muscle tissue in complete Freund's adjuvant. A boosting injection with the specific antigens three weeks after the initial immunisation did not markedly increase the incidence of thymitis or of partial neuromuscular block.

In animals with a partial neuromuscular block, thymectomy was followed by return of the muscle tetanus pattern to normal within two to three days. In contrast, the partial neuromuscular block persisted for four days after hemithymectomy and in some animals for as long as 10 days after sham thymectomy.

These findings are in keeping with the hypothesis that experimentally induced thymitis may result in the release of a factor from the thymus which blocks neuromuscular transmission.

### INTRODUCTION

An experimental model of human myasthenia gravis can be induced in guinea-pigs or rats by immunisation with either thymus or skeletal muscle homogenates emulsified in complete Freund's adjuvant (CFA) (Goldstein & Whittingham, 1966, 1967; Goldstein & Hofmann, 1968; Goldstein, Strauss & Pickeral, 1969; Kalden *et al.*, 1969; Kalden & Irvine, 1969). Affected animals show both thymitis and a partial neuromuscular block similar to that of human myasthenia gravis.

It has been demonstrated (Goldstein & Whittingham, 1966; Kalden *et al.*, (1969) that the development of the partial neuromuscular block in the experimental animals is dependent on the presence of thymitis, and that thymectomy prior to immunisation prevents the development of a block in neuromuscular transmission.

To establish further the pathogenic importance of the thymus in relation to the partial neuromuscular block in experimental myasthenia gravis, it was decided to investigate the effect of thymectomy, hemithymectomy and sham thymectomy in guinea-pigs with electromyographic evidence of a partial neuromuscular block occurring 14-16 days after the immunisation with homologous thymus, skeletal muscle or heart muscle in CFA.

## MATERIALS AND METHODS

### Animals

Female Hartley guinea-pigs of 400-500 g. body weight derived from an outbred strain from animal suppliers (London Ltd.) were used for immunisation. Tissue antigens were prepared from young Hartley guinea-pigs weighing about 100 g.

### Antigen preparation

Fresh thymus, skeletal muscle and heart muscle tissue were homogenised (20% w/v) in phosphate buffered saline pH 7.2. The homogenates were centrifuged for 15 minutes at 2500 g. and the supernatants lyophilised. The thymus and skeletal muscle tissue powders were prepared for injection by dissolving the lyophilisates in distilled water to a concentration of 50 mg./ml. The tissue extracts and phosphate buffered saline, respectively, were emulsified with equal volumes of complete Freund's adjuvant (CFA) (Difco.).

### Immunisation

Each animal was injected with 0.1 ml. emulsion into each hind foot pad. Fifteen animals immunised with thymus, and 15 animals immunised with skeletal muscle (both in CFA) received tissue doses of 5 mg. Eight animals immunised with heart muscle in CFA received a tissue dose of 8 mg. Fifteen control animals were injected with phosphate buffered saline emulsified



in CFA (fig. 1a, Table 1).

Guinea-pigs which did not show symptoms of a partial neuromuscular block 14 days after the initial immunisation received the same amount of the corresponding antigen in CFA three weeks after the initial immunisation. Nine control animals received a boosting injection with saline in CFA (fig. 1b).

#### Immunofluorescence test

The indirect immunofluorescence test (Weller & Coons, 1954) was used. Undiluted serum or serum dilutions 1:8 were applied to unfixed air-dried cryostat sections of guinea-pig thymus, skeletal muscle and heart muscle for 20 minutes. The sections were then washed in veronal buffer (pH 7.2) for 30 minutes.

A polyvalent rabbit anti-guinea-pig gamma globulin serum (Hyland Division, Travenol Laboratories, Los Angeles) was conjugated with fluorescein isothiocyanate isomer 1 (British Drug Houses Ltd., Poole, Dorset, England) and subsequently dialysed as previously described (Kalden *et al.*, 1969). The protein-fluorescein ratio was found to be 0.5. The fluorescein conjugated antiserum was applied in a dilution 1:8 for 20 minutes. After a final wash for one hour in veronal buffer (pH 7.2) the sections were mounted in 10% glycerol in saline and examined using a Gillet and Sibert microscope with a 100V iodine-quartz lamp with primary filter 30/063 and ocular filter 10/285.

#### Histology

Specimens of thymus, diaphragm and heart muscle were fixed overnight in Carnoy solution and sections were prepared in the usual way and stained with hematoxilin and eosin. Slides were read without knowledge of the groups to which the animals belonged. A marked degree of lymphocytic aggregation around the Hassall's corpuscles in the thymic medulla was interpreted as thymitis (fig. 2a).



### Electromyography

Electromyographic recordings were made in both front limbs. For stimulating and for recording, a pair of Copeland-Davies clip electrodes were used (1 cm. across the clip) supplied by Electro - Physiological Instruments Ltd., Edinburgh. The stimulating electrodes were clipped through the shaved skin of the axilla so that the tips of the clips were close to the median nerve. The recording electrodes penetrated the unexposed flexor digitorum through the shaved skin and were placed as close together as possible. In each animal at least three recordings from three different muscle areas were made.

The nerve stimulation was supra-maximal with a pulse duration of 100 m.sec. In each instance a single compound muscle action potential was observed at a sweep speed of 2 m.sec./cm. For tetanus, a stimulation frequency of 50/sec. was used and the first ten muscle action potentials observed at a sweep speed of 20 m.sec./cm. The first 25 muscle action potentials were observed at a sweep speed of 50 m.sec./cm.

A double pulse unit (2 GIRO, Electro-Physiological Instruments Ltd., Edinburgh) was used for stimulation. The electromyographic response was recorded with a 'Tektronix 564' storage oscilloscope with a '2B67' time base and a '3A72' dual trace amplifier unit. Representative pictures were taken with a 'Tektronic C-12 Land Polaroid' camera (Tektronic Inc., Portland, Oregon).

### Plan of experiments

Fourteen to sixteen days after the initial immunisation all the guinea-pigs were anaesthetized with Nembutal 35 mg/kg body weight.

The 19 animals (fig. 1) which showed a neuromuscular block were divided into three groups. Six of these animals were thymectomised at day 15 and the electro-myographic studies repeated on the second and third day after the operation.

Five of the animals were hemithymectomised and eight were sham thymectomised at day 16 after the initial immunisation. In both these groups electromyography was repeated at days four and ten after the operation.

The controls, immunized with saline in CFA, comprised six thymectomized animals and nine unoperated animals.

In the case of the animals which did not show a partial neuromuscular block 14-16 days after the initial immunisation, the electromyographic studies were repeated one week after the boosting injection.

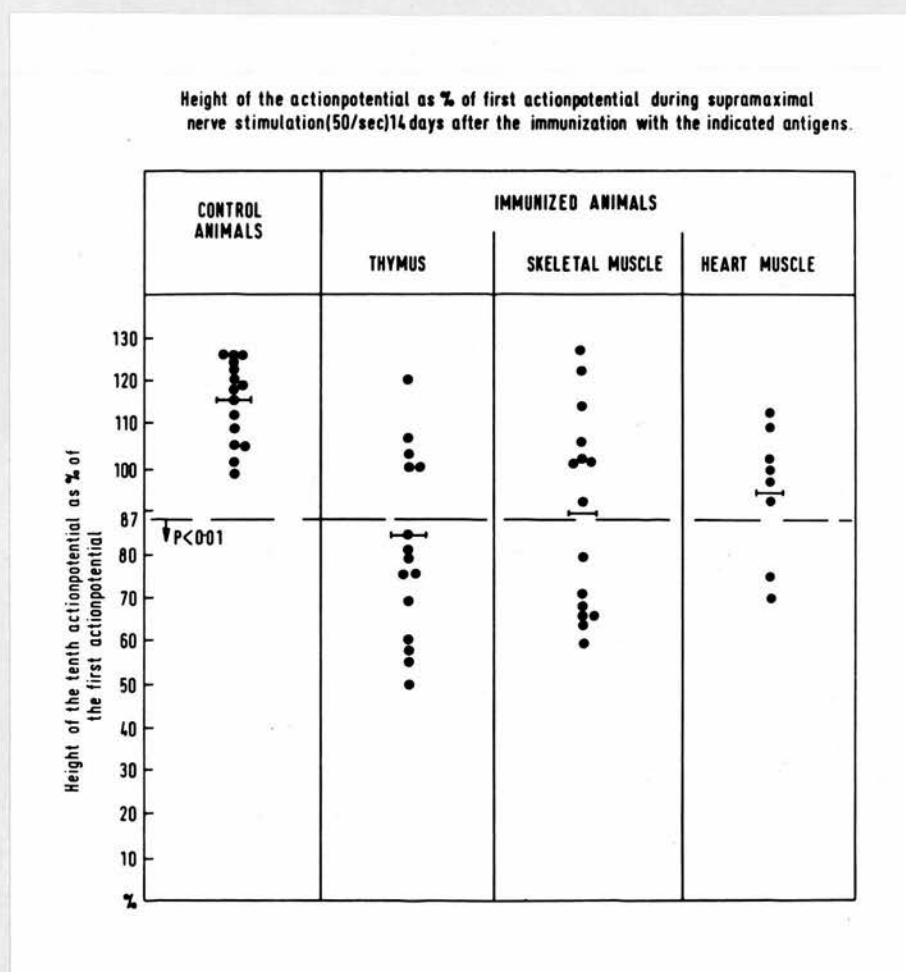
After the final electromyographic study the animals were killed with chloroform and bled. The sera were stored at  $-20^{\circ}\text{C}$ .

## RESULTS

### Incidence of neuromuscular block

The behaviour of the first ten muscle action potentials during supra-maximal tetanic nerve stimulation was used to study the neuromuscular transmission. The ratio of the amplitude of the tenth to the first action potential was noted. From the recordings in the 15 control animals a decline in the height of the tenth action potential to less than 87% of the first one seemed to be unlikely ( $P < 0.01$ ). Therefore 10 out of 15 guinea-pigs immunised with thymus in CFA, 7 out of 15 immunised with skeletal muscle in CFA and 2 out of 8 immunised with heart muscle in CFA were regarded as having abnormal neuromuscular transmission (fig. 1a).

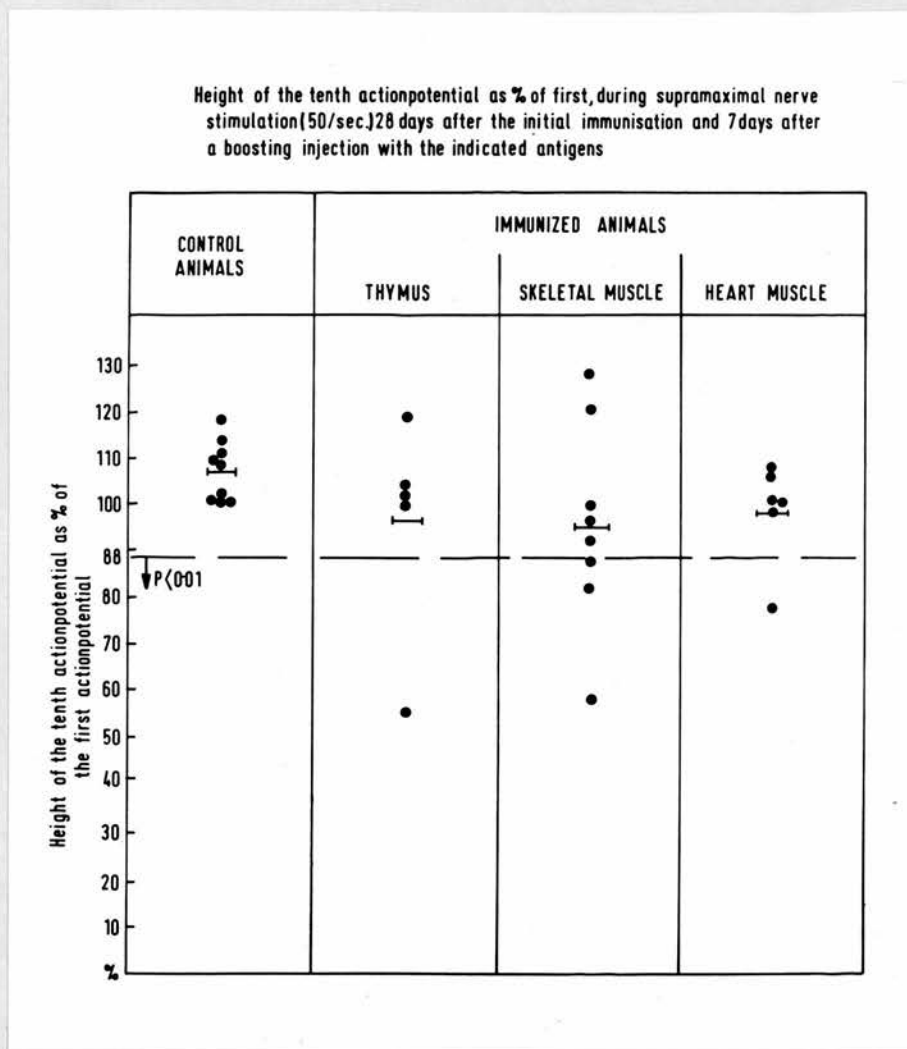
Of those animals which did not show a disturbance in the neuromuscular transmission 14 days after the initial immunisation and which had received a boosting injection on day 21, 1 out of 5 immunised with thymus, 3 out of 8 immunised with skeletal muscle and 1 out of 6 immunised with heart muscle were found to have a significant decline ( $P < 0.01$ ) in the successive muscle responses one week after the boosting injection (fig. 1b).



**Fig. 1a**

Electromyographic response to supra-maximal nerve stimulation at a rate of 50/sec. The individual results shown are the means of the readings in each front limb

a) 15 days after immunisation. From the recordings in the control animals a decline in the height of the tenth action potential to less than 87 per cent of the first was unlikely ( $P \leq 0.01$ ). Therefore, 10 of the guinea-pigs immunised with thymus, 7 immunised with skeletal muscle and 2 immunised with heart muscle were considered to have a disturbance in neuromuscular transmission.

**Fig. 1b**

The same as Fig. 1a but one week after a boosting injection on day 21 in animals without electromyographic evidence of a partial neuromuscular block 15 days after the initial immunisation. The lower confidence limit in the control animals was 88 per cent. Note that a boosting injection was followed by a significant decline in successive muscle responses only in one animal immunised with thymus, three animals immunised with skeletal muscle and one guinea-pig immunised with heart muscle.



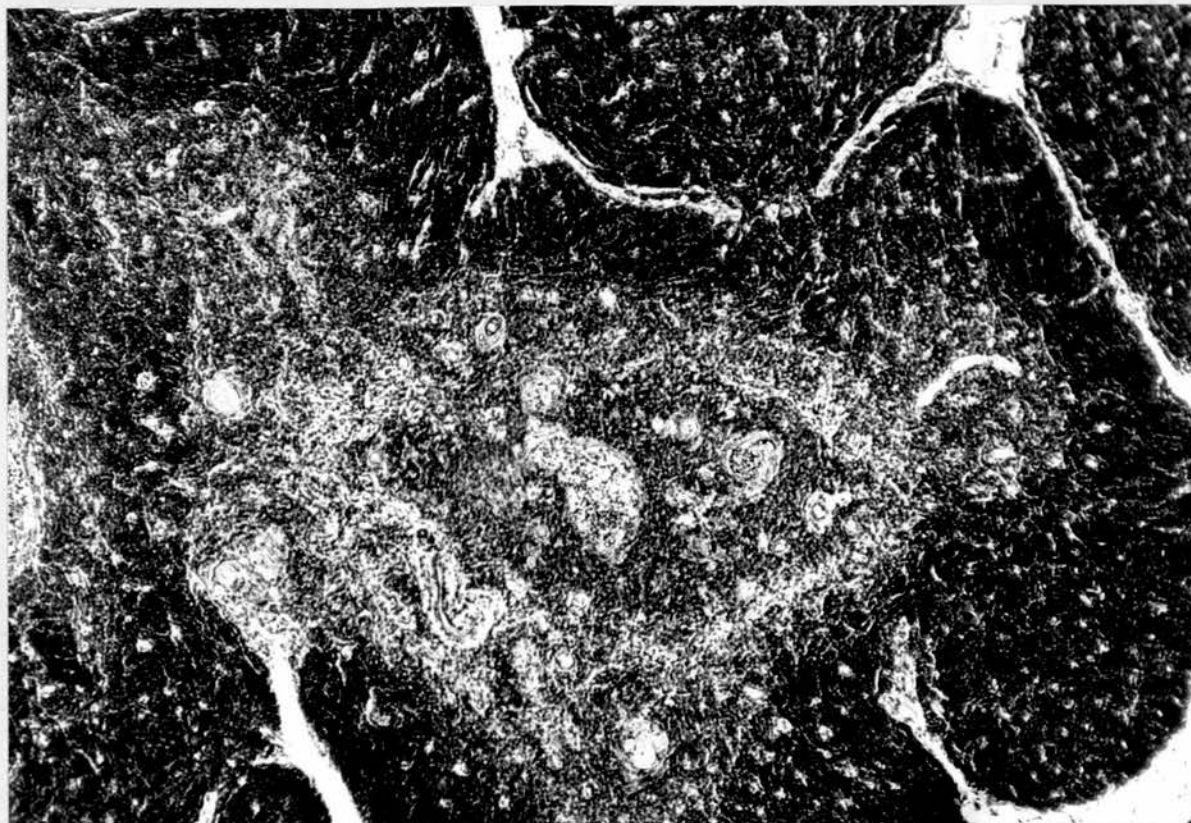


Fig. 2a + b

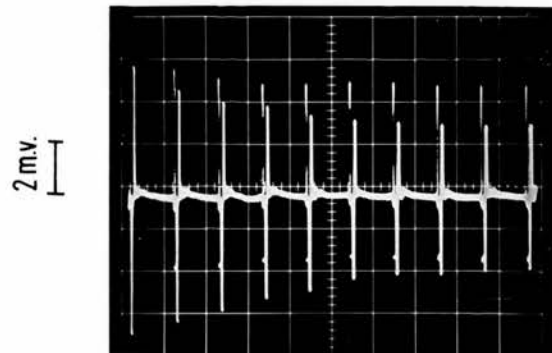
Thymus histology and electromyographic record of a guinea-pig immunised with homologous thymus tissue in C.F.A.

a) Thymus histology 15 days after immunisation. Note the dense lymphocytic infiltration around the Hassall's corpuscles in the medulla. This histological appearance is referred to as thymitis.

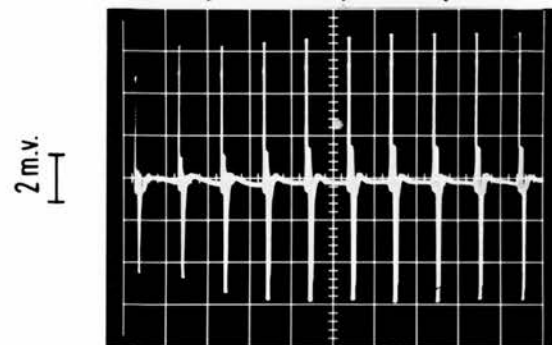
H & E x 100

ELECTROMYOGRAPHIC RESPONSE OF GUINEA-PIG FOREARM  
MUSCLE TO SUPRAMAXIMAL NERVE STIMULATION AT A  
RATE OF 50/sec

A. Before Thymectomy



B. Three Days after Thymectomy



20m. sec

Fig. 2b

Muscle action potentials in the flexor digitorum following supra-maximal nerve stimulation at a rate of 50/sec. Note that before thymectomy on day 15 after immunisation, the tenth muscle action potential is 53.1 per cent. of the first in contrast to the normal muscle tetanus pattern three days after thymectomy.

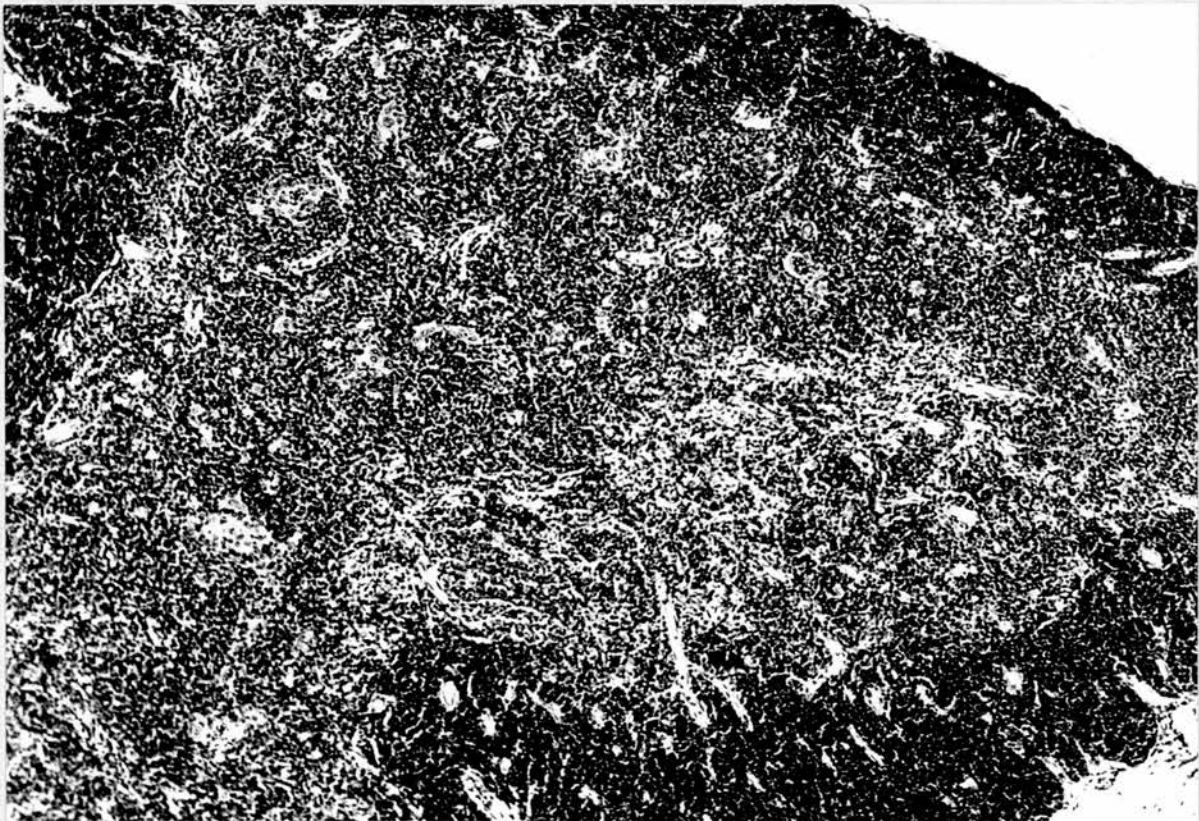


Fig. 3a + b

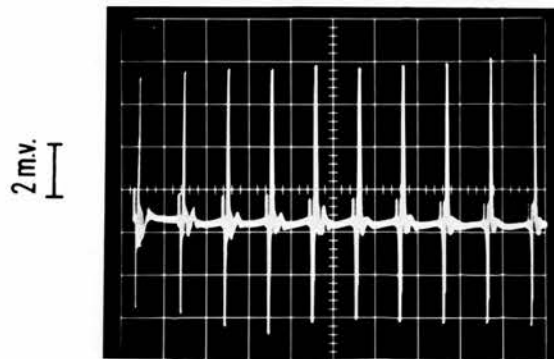
Thymus histology of electromyographic record of a control animal immunised with saline in C. F. A.

a) In contrast to Fig. 2a normal guinea-pig thymus showing a comparatively even distribution of lymphocytes throughout the thymic medulla.

H & E x 100.

ELECTROMYOGRAPHIC RESPONSE OF GUINEA-PIG FOREARM  
MUSCLE TO SUPRAMAXIMAL NERVE STIMULATION AT A  
RATE OF 50/sec

A. Before Thymectomy



B. Three Days after Thymectomy

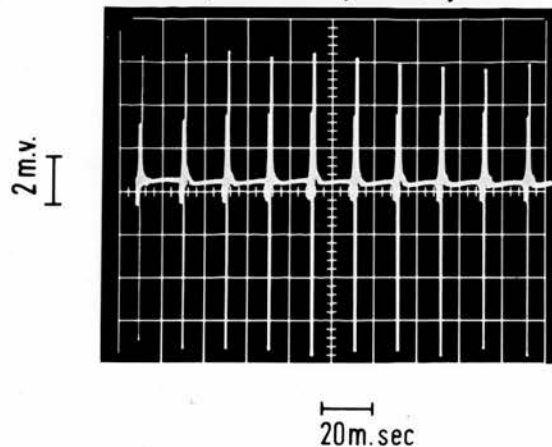
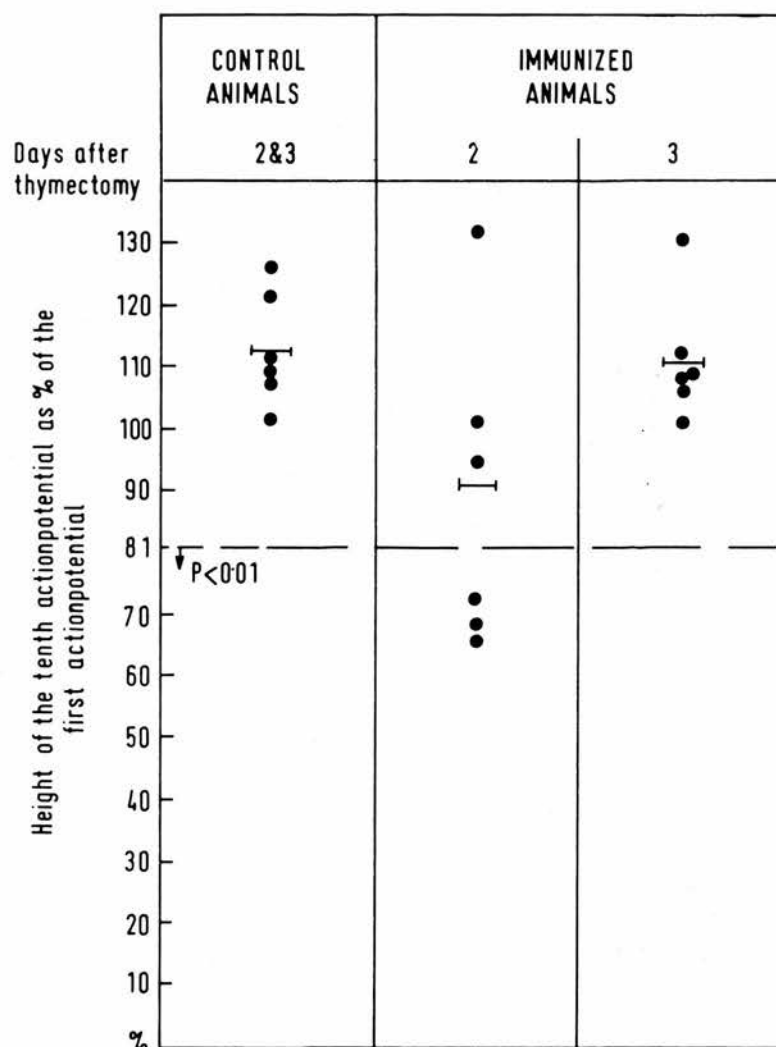


Fig. 3b

Muscle action potentials in the flexor digitorum following supra-maximal nerve stimulation at a rate of 50/sec. In contrast to Fig. 2b note the normal muscle tetanus pattern before and after thymectomy.

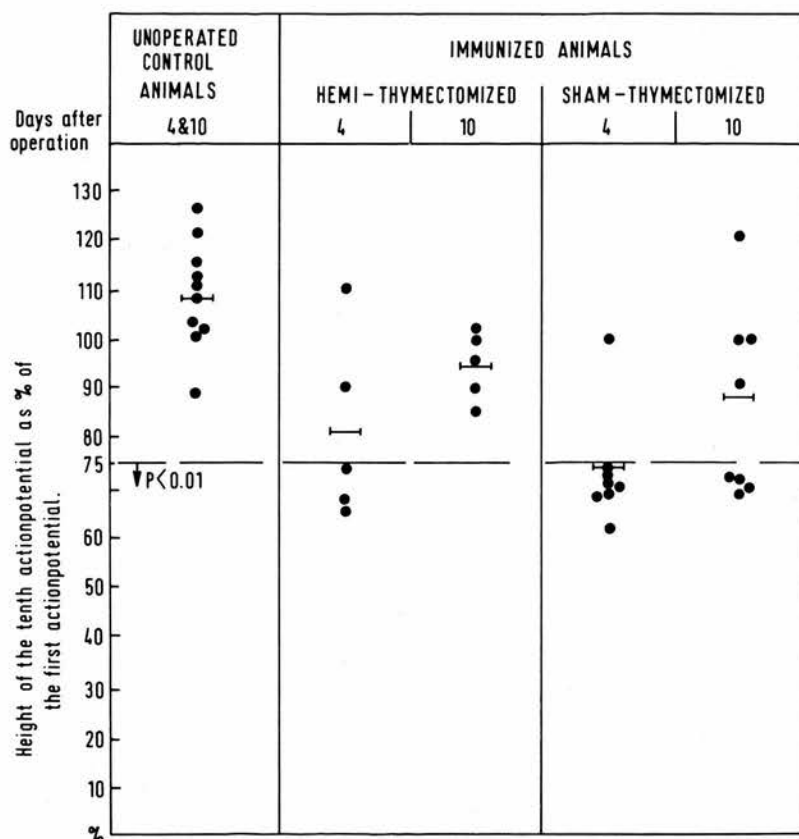




**Fig. 4a + b**

The effect of thymectomy (day 15), hemithymectomy (day 16) and sham thymectomy (day 16) on the neuromuscular transmission of guinea-pigs with evidence of a partial neuromuscular block at day 15 after the antigen injection. For each individual control animal the mean of the recordings made on each of the different post-operative days is shown. The nerve stimulation was always supra-maximal at a rate of 50/sec.

a) Electromyographic recordings two and three days after thymectomy in six control animals and six immunised animals. The lower confidence limit in the control animals was 81 per cent. Note that within either two or three days the muscle tetanus pattern of the animals with a partial neuromuscular block had returned to normal.

**Fig. 4b**

Electromyographic recordings four days and ten days after a hemithymectomy or sham thymectomy. From the recordings in the unoperated controls, a decline in the height of the tenth action potential to less than 75 per cent. of the first was unlikely ( $P \leq 0.01$ ). In contrast to Fig. 4a three of the hemithymectomised animals and seven of the sham thymectomised animals still showed electromyographic evidence of a partial neuromuscular block after four post-operative days. In four sham thymectomised animals a partial neuromuscular block was still demonstrable ten days after the operation.

TABLE 1  
EXPERIMENTAL THYMITIS IN GUINEA PIGS OBSERVED AT DIFFERENT TIMES  
AFTER THE IMMUNISATION WITH INDICATED ANTIGENS

Antigen injected in C.F.A.	No. of animals	Incidence of thymitis on day <sup>x</sup>					Total incidence	Significance $\chi^2$ -test
		15	16	26	26	28		
		total thymectomy	hemithymectomy	hemithymectomy	sham thymectomy	boosting injection <sup>xx</sup>		
			1st lobe	2nd lobe	on day 16	on day 21		
Thymus	15	3/3	3/3	2/3	3/4	1/5	10/15	$p < 0.02$
Skeletal muscle	15	2/2	2/2	0/2	2/3	4/8	10/15	$p < 0.02$
Heart muscle	8	1/1	-	-	0/1	2/6	3/8	N.S. +
Saline	15	1/6	-	-	-	2/9	3/15	-

x after the initial immunisation

xx same volume as used for the initial immunisation

+ not significant

### The effect of thymectomy, hemithymectomy and sham thymectomy

As shown in fig. 2b and 4a thymectomy in all six animals with a partial neuromuscular block was followed by a complete reversion of the tetanus pattern to a normal picture within either two or three days. No changes were found in the muscle tetanus pattern before and after thymectomy in the control animals (fig. 3b and fig. 4a).

In the case of the five guinea-pigs with a partial neuromuscular block which were hemithymectomised 16 days after the initial immunisation, three animals were still showing four days after the operation a significant decline in the successive muscle responses to supra-maximal nerve stimulation (fig. 4b). However, 10 days after hemithymectomy the muscle tetanus pattern had returned to normal in all the guinea-pigs.

Seven of the eight animals with a partial neuromuscular block which had been sham thymectomised 16 days after immunisation still showed evidence of a partial neuromuscular block 4 days later. In 4 of these the partial neuromuscular block was still present 10 days after sham thymectomy (fig. 4b).

The mean amplitudes of a compound single action potential to supra-maximal nerve stimulation recorded in the controls and in the different groups of immunised animals before and after thymectomy, hemithymectomy, sham thymectomy or a boosting injection were not significantly different from each other ( $P$  always  $> 0.05$ ).

### Incidence of thymitis

Table 1 shows the incidence of thymitis in the immunised and control guinea-pigs obtained at different times after the primary antigen injection or 1 week after a boosting injection on the 21st day after the initial immunization. The incidence of thymitis in 10 out of 15 animals immunised with thymus in CFA and in 10 out of 15 animals immunised with skeletal muscle in CFA is significantly higher ( $P < 0.02$ ) than the incidence in the control animals (3 out of 15). The occurrence of thymitis in 3 out of 8 animals



immunised with heart muscle in CFA was not significant when compared with that in the control animals.

#### Correlation between partial neuromuscular block and thymitis

All the immunised guinea-pigs with a partial neuromuscular block which were thymectomised or hemithymectomised 15 and 16 days after the immunisation showed histological evidence of thymitis. However, although by day 26 (which is 10 days after the operation) none of the 5 hemithymectomised animals had evidence of a partial neuromuscular block (fig. 4b), thymitis was still present in the second thymus lobe in two of these animals (Table 1). Thymitis was present in all four of the eight sham thymectomised guinea-pigs which still had evidence of a partial neuromuscular block 10 days after the operation (i.e. 26 days after the immunisation) (fig. 4b).

In animals with no evidence of abnormal neuromuscular transmission 14 days after the primary antigen injection a boosting injection three weeks after the initial immunisation induced a thymitis in 1 out of 5 immunised with thymus, 4 out of 8 immunised with skeletal muscle and in 2 out of 6 immunised with heart muscle (Table 1). With the exception of one animal immunised with skeletal muscle and one immunised with heart muscle all the guinea-pigs with thymitis also developed a partial neuromuscular block (fig. 1b).

#### Muscle histology

One of the animals immunised with thymus showed a focal inflammatory reaction in the diaphragm and one of the animals immunised with skeletal muscle and one immunised with heart muscle showed a myositis in the diaphragm and a focal myocarditis. A focal myocarditis was also found in three guinea-pigs which has been immunised with heart muscle and which had received a boosting injection. No abnormalities in skeletal muscle or myocardium were noted in the remaining test or control animals.

### Immunofluorescence for serum antibodies

Undiluted serum from nearly all the test and control animals gave an immunofluorescence staining of thymus lymphocytes and of the cross striations of skeletal and heart muscle. When the sera were diluted 1:8 only 3 out of 15 thymus immunised animals gave a definite reaction with the cytoplasm of thymus lymphocytes and 3 out of 15 skeletal immunised guinea-pigs with the cross striations of skeletal muscle and of heart muscle and with the cytoplasm of thymus myoid cells. Four out of eight animals immunised with heart muscle showed only a weak reaction with the cross striations of heart muscle tissue when the sera were diluted 1:8. None of the sera of the control animals showed positive immunofluorescence at this dilution. Positive controls were performed using guinea-pig sera with a high titre of skeletal muscle or thymus lymphocyte antibodies.

### DISCUSSION

Immunisation with either homologous or heterologous thymus or skeletal muscle homogenates in CFA has been shown to be effective in inducing a thymitis and a partial neuromuscular block in guinea-pigs and rats (Goldstein & Whittingham, 1966, 1967; Goldstein et al., 1969; Kalden et al., (1969). The autoimmune nature of the experimental thymitis has been further established by the production of thymitis in inbred Lewis rats immunised with histo-compatible Lewis rat thymus (Goldstein and Hofmann, 1968). The effectiveness of skeletal muscle and of heart muscle as antigens in the production of thymitis has been explained on the basis of a common antigen in skeletal muscle, heart muscle and thymus myoid cells (Van der Geld & Strauss, 1966, Beutner et al., 1962).

The development of abnormal neuromuscular transmission in the immunised animals has been shown to be dependent on the presence of thymitis (Goldstein and Whittingham, 1966, Kalden et al., 1969). It has been

suggested that the partial neuromuscular block in the immunised animals is caused by a factor secreted from a thymus which is the site of an immunopathogenic thymitis. Goldstein and Hofmann (1969) have published experimental evidence showing that the thymus may secrete a factor which may have physiological importance in the regulation of neuromuscular transmission. However, attempts to extract from thymus tissue a substance influencing neuromuscular transmission have so far produced only equivocal results (see Goldstein, 1968, for references).

In the present experiments, evidence for a partial neuromuscular block in test and control animals was looked for by electromyography using Copeland-Davies clip electrodes as the recording system. In this system the parameter for detecting a partial neuromuscular block is the recording of the muscle tetanus pattern during supra-maximal nerve stimulation. Since the number of single muscle fibre units picked up during a recording is dependent on the particular location of the clip electrodes, the height of a single compound muscle action potential does not give any specific information about a pathological change in the neuromuscular transmission.

The results presented in this paper give further support to the hypothesis that the thymus which is the site of an immunologically induced thymitis may indeed be the source of a neuromuscular blocking factor. Thymectomy in animals with a disturbance in neuromuscular transmission 15 days after immunisation was followed by reversion of the muscle tetanus pattern to normal within two or at most three days. In contrast, the block persisted for at least four post-operative days in all the animals subjected to hemi or sham thymectomy and for 10 post-operative days in half of the sham thymectomised animals.

In human myasthenia gravis thymectomy is most likely to be beneficial in young female patients with thymitis (referred to as thymic dysplasia by Goldstein, 1966) and when the operation is performed shortly after the onset of the disease (Simpson, 1958; Keynes, 1954). Thymectomy



is likely to be of little help if the duration of the disease has been long with irreversible structural changes in the motor endplates (Woolf, 1966), or in the muscle fibres (Fenichel, 1966) or if ectopic thymus tissue is present (Harvey, 1948; Fisher, 1964).

The fact that only half of the sham thymectomised animals still showed a neuromuscular block 10 days after the operation (that is 26 days after the immunisation) correlates with the transience of experimental thymitis. The brief duration of experimental thymitis produced by immunisation with either thymus tissue or skeletal muscle tissue in CFA is comparable to that of experimental thyroiditis (Jones & Roitt, 1961).

No attempt was made to grade the severity of thymitis within the medulla of the gland. The number of histological sections of each thymus was limited and it is therefore possible that a focal lesion may have been considered to be generalised. This is the most likely explanation for the fact that not all the animals with histological evidence of thymitis had a partial neuromuscular block. It may also explain why histological evidence of thymitis was present 10 days after the operation in one of the 8 sham thymectomised guinea-pigs, although in these guinea-pigs the muscle tetanus pattern had returned to normal.

As reported in relation to other experimental animal models of auto-allergic disease, such as experimental adrenalitis (Andrada et al., 1968), immunisation of the guinea-pigs with one injection of thymic tissue in CFA seemed to suffice for the development of a high incidence of thymitis and subsequently of a neuromuscular block (Table 1, fig. 1). In the case of immunisation with skeletal muscle tissue in CFA, however, 4 out of 8 guinea-pigs developed thymitis after a boosting injection three weeks following the initial antigen injection. Three of these animals had evidence of a neuromuscular block. One explanation for this enhancing effect of the boosting injection could be the smaller amount of antigen reactive with thymus in skeletal muscle tissue used for the initial immunisation (Goldstein et al., 1969)



In the present experiments homologous tissue extracts were used for immunisation and only weak antibody titres were found in a small number of the immunised animals. These results, which demonstrate the lack of correlation between the incidence of experimental thymitis and circulating auto-antibodies, are in keeping with other animal experimental models (Terplan et al., 1961; Andrada et al., 1968; Davies et al., 1964).

Some recent publications (Vetters et al., 1969; Kaufman et al., 1969) deny that a myasthenia-like syndrome can be induced in experimental animals by immunological techniques. However, these papers do not give a histological description of thymitis. According to the hypothesis discussed above a partial neuromuscular block would not be expected in the absence of experimental thymitis.

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The appearance of immunological competence at an early age in  
New Zealand black mice

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## THE APPEARANCE OF IMMUNOLOGICAL COMPETENCE AT AN EARLY AGE IN NEW ZEALAND BLACK MICE

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### SUMMARY

Adult and 5-day-old New Zealand Black (NZB) and Ju control strain mice were injected with sheep red blood cells. At various times after injection they were killed and the spleens, lymph nodes and thymus tested for haemolysin-producing cells. When the response was expressed as plaque-forming cells (PFC) per million viable cells, the response curve of the spleens of baby NZB mice was very similar to the response in the spleens of the adults, and the response in the lymph nodes of the babies was as high and more sustained than that of the adults. In Ju control strain baby mice of this age, the response in the spleen and the lymph nodes was both reduced and delayed compared with the adults. Neither strain gave a significant response in the thymus. The spleen and lymph nodes of adult NZB mice showed a response which was delayed but not reduced as compared with the adults of the Ju control strain, whereas in baby NZB mice the spleen and lymph nodes showed a response which was advanced and increased (particularly in the lymph nodes) compared with control strain babies (Ju, Swiss, C57Bl, CBA). The NZB mice did not reach this level of responsiveness until they were 4–5 days old.

### INTRODUCTION

New Zealand Black (NZB) mice are a strain developing spontaneous autoimmune haemolytic anaemia and other manifestations of autoimmunity (Helyer & Howie, 1963; Holmes & Burnet, 1963; Howie & Helyer, 1965; Mellors, 1966a). They provide a useful experimental system for the study of idiopathic autoimmune disease. Autoimmune disorders may, theoretically, be caused by defects or abnormalities in the antibody-forming system, and in NZB mice structural abnormalities of the thymus (Abbot & Burnet, 1964; Burnet & Holmes, 1964; Siegler, 1965), spleen (Holmes & Burnet, 1963; East, de Sousa & Parrott, 1965) and the lymphoid system in general (East *et al.*, 1965; Mellors, 1966b) suggest this as an explanation.

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Functional abnormalities of the antibody-forming system have been investigated by studying the response of the animal to heterologous antigens. Diener (1966) has found that the spleens of adult NZB show a reduced and delayed response to sheep red blood cells compared with a control strain. Playfair (1967) in similar experiments with baby NZB found an increased response compared with controls. The present experiments explore these results further, in particular comparing the response curve of the babies in spleen, lymph nodes and thymus with that of the adults.

## MATERIALS AND METHODS

### *Animals*

NZB mice have been maintained at M.R.C. Clinical Endocrinology Research Unit for five generations. They are a stock which was obtained from the Department of Surgical Science, University of Edinburgh, where they had been bred for five generations since being obtained from Dr M. Bielschowsky in New Zealand. They have been inbred by sibling matings for over sixty generations altogether.

Ju mice, which were used as a control strain, are also maintained at CERU. They are a stock developed by the Department of Animal Genetics, University of Edinburgh, and have been inbred by sibling matings for over forty generations. They do not produce autoantibodies to red blood cells.

The Swiss mice used in this study were a non-inbred closed colony maintained by CERU.

C57Bl and CBA mice were kindly provided by Dr J. Carr of the British Empire Cancer Campaign.

### *Antigen challenge*

Adult mice were 150–320 days old when challenged at which time many of the NZB animals showed serum anti-red cell autoantibodies detectable by the Coombs test. Adult mice were injected intraperitoneally with 0.25 ml (approximately  $4 \times 10^9$ ) washed, packed sheep red blood cells (SRBC) and were killed between 2 and 8 days after injection.

Baby mice were challenged at 5–6 days old, except where stated otherwise. They were injected intraperitoneally with 0.025 ml washed, packed SRBC, and were killed 3–9 days after injection. Mice represented on the figures as being killed at 0 days were uninjected controls.

### *Detection of antibody-forming cells*

The mice were killed with ether, partially bled out and the spleens, limb-draining lymph nodes and, in some animals, the thymus, were removed, placed in ice-cold Eagle's medium and disrupted by gentle homogenization in a Griffith tissue grinder. The viable cells, identified by using a trypan blue exclusion test, were counted on a haemocytometer. A slightly modified form of the Jerne, Nordin & Henry (1963) technique was used for the detection of haemolytic plaque-forming cells (PFC). 0.1 ml of lymphoid cell suspension, 1 drop 1:15 v/v SRBC and 1 drop preserved guinea-pig serum (Stayne's) were added to 0.5 ml 0.6% agar in Eagle's medium at 47°C and immediately spread over a glass slide on a level surface. When the agar had set, the slides were incubated at 37°C for 2 hr and the number of plaques counted under a dissecting microscope in dark field. Where the volume of cell suspension allowed, five replicates were done for each tissue.

*Analysis of results*

Logarithmic means were calculated for each point (0 being treated as 1) and the standard error is shown. Where significant differences are stated they are at the 5% level and have been calculated by the randomization method. Regression lines were plotted for Fig. 4, and the significance of the differences in the regression coefficients was determined at the 5% level.

## RESULTS

Fig. 1 shows the response curves of the spleen of both strains of adult mice and 5-day-old baby mice injected with antigen. The responses of the adult and baby NZB mice both rose

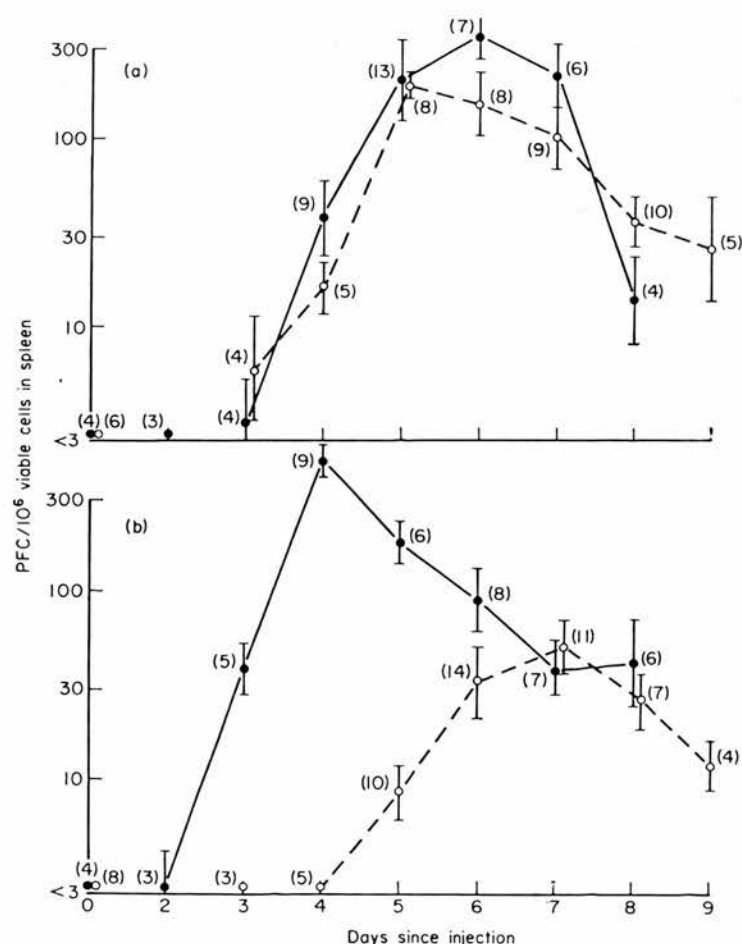


FIG. 1. The immune response of the spleen, expressed as plaque-forming cells (PFC)/10<sup>6</sup> viable cells. (a) NZB, and (b) Ju mice. Adult mice (●) were injected with 0.25 ml packed washed SRBC. Baby mice (○) were injected with 0.025 ml packed washed SRBC at 5 days old. The mice were killed 2-9 days after injection. The points are logarithmic means, the limits represent the standard error and the numbers in parentheses indicate the number of animals.



to a maximum at 5–6 days after injection, the adults producing a log mean response of 363 PFC/ $10^6$  viable cells and the babies 194 PFC/ $10^6$ . There was no significant difference between the adults and babies at 5, 6 or 7 days after injection, showing that the response of the baby NZB mouse was very similar to that of the adult NZB. The peak response of the adult Ju mice occurred 4 days after antigen challenge with a mean of 480 PFC/ $10^6$  viable

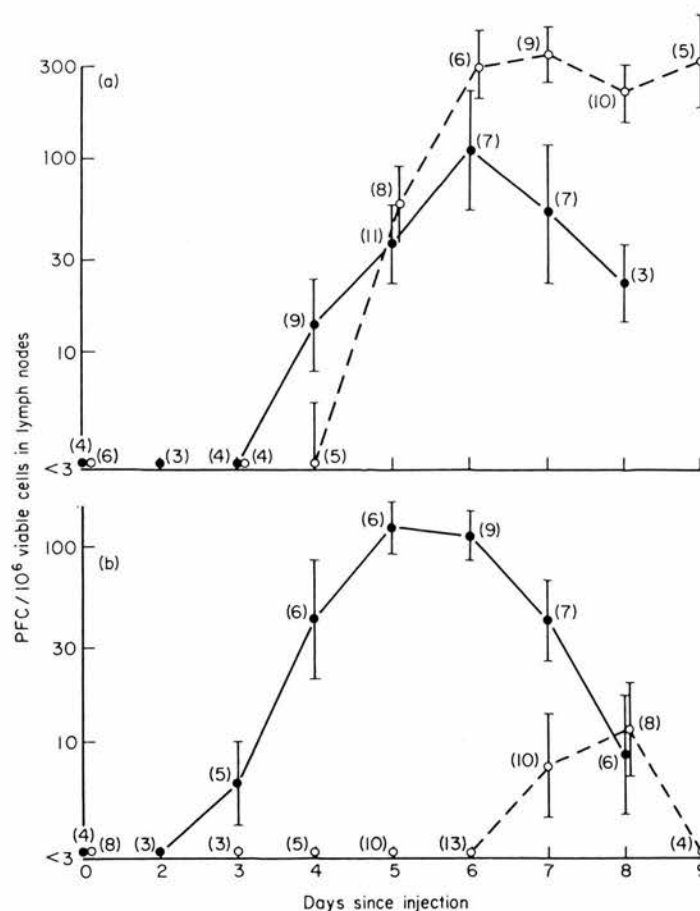


FIG. 2. The immune response of the lymph nodes, expressed as PFC/ $10^6$  viable cells. (a) NZB, and (b) Ju mice. Adult mice (●) were injected with 0.25 ml packed washed SRBC. Baby mice (○) at 5 days old were injected with 0.025 ml packed washed SRBC. The mice were killed 2–9 days after injection. The points are logarithmic means, the limits represent the standard error and the numbers in parentheses indicate the number of animals.

cells, while the babies did not reach their peak response until 7 days after antigen challenge when they gave a mean of only 49 PFC/ $10^6$  viable cells—one-tenth that of the adults. This difference is significant. The adult NZB reached their maximum response 1–2 days later than the Ju adults, but the difference in the peak heights is not significant. The baby NZB mice showed their maximum response 1–2 days before the baby Ju mice and the peak is

significantly higher. The number of plaques produced by the uninjected Ju mice was negligible. The number of plaques produced by the uninjected NZB mice, both adults and babies, was greater than that of the Ju mice but in only two cases (one adult and one baby) was the number of PFC greater than  $3/10^6$  viable cells.

Fig. 2 shows the response of the lymph nodes. The adult NZB mice showed the peak of their response at 6 days after antigen challenge with a mean of 110 PFC/ $10^6$  viable cells and the response of the baby NZB mice reached a plateau at 6 days with a maximum of

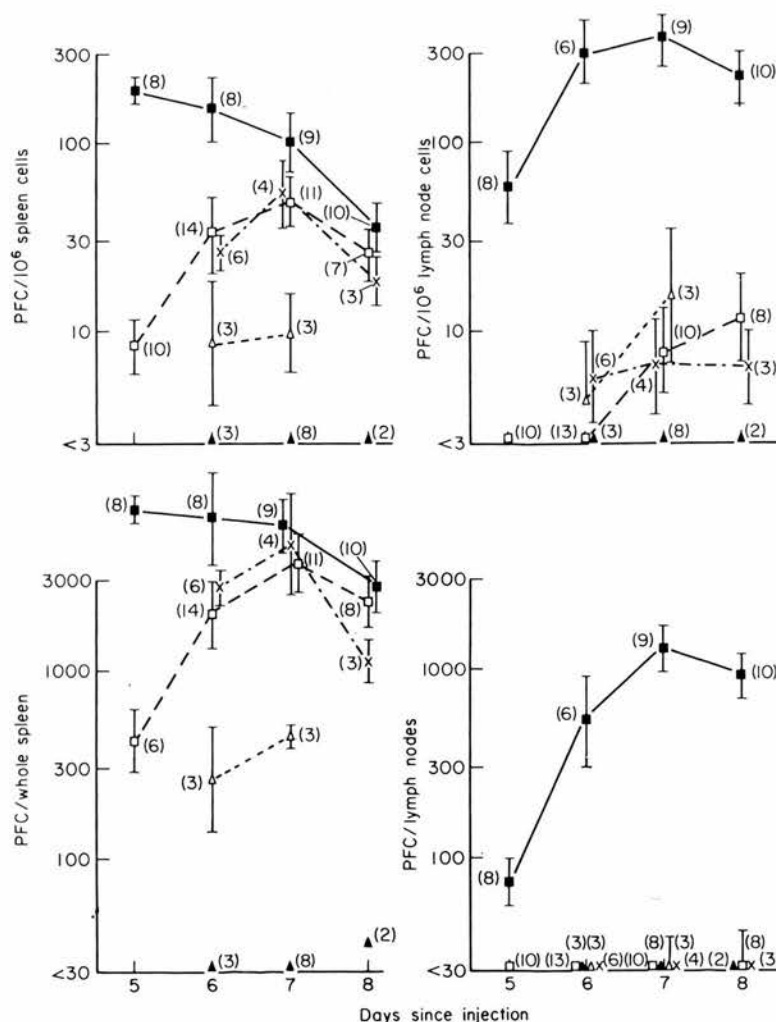


FIG. 3. The immune response of the spleens and lymph nodes of various strains of baby mice: ■, NZB; □, Ju; ×, Swiss; ▲, C56B1; △, CBA. They were injected at 5 days old with 0.025 ml packed washed SRBC and killed at various times after injection. The results are expressed either as the number of PFC/ $10^6$  viable cells in the organ or as the number of PFC per whole spleen or per the dissected lymph nodes (the same ones in all cases) from each animal. The points are logarithmic means, the limits represent the standard error and the numbers in parentheses indicate the number of animals.

355 PFC/ $10^6$  viable cells and this number was not reduced by 9 days. The difference in the height of the response of the adult and baby NZB mice at 6 days is not significant, but the difference at 7 and 8 days is. The adult Ju mice reached their peak response at 5 or 6 days after the challenge with a mean of 126 PFC/ $10^6$  viable cells, while the baby Ju mice did not reach their peak until 8 days with a mean of only 11 PFC/ $10^6$ . This difference is significant. There is also a significant difference between the heights of the peaks of the adult Ju and NZB at 5 days, but none at 6 days. The peak in antibody production in the baby NZB mice occurred 1–2 days before that in the baby Ju mice and the peak in the baby NZB mice was significantly higher. The lymph nodes of the uninjected mice gave negligible responses in all cases.

In many of these mice the thymus was also tested, but in no case was there more than a very slight increase in the numbers of PFC and in the few cases where this occurred it could be explained by accidental inclusion of small amounts (under 10%) of tissue from the circumaortic lymph nodes.

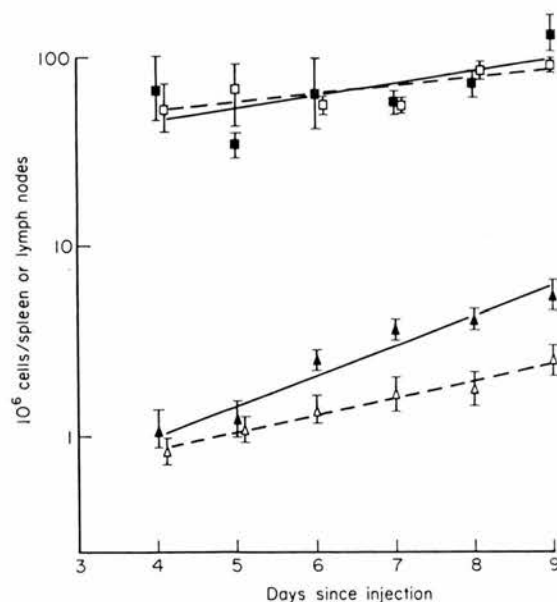


FIG. 4. The number of viable cells in the spleens and dissected lymph nodes (the same ones in all cases) of baby NZB and baby Ju mice. They were injected at 5 days old with SRBC and killed at various times after injection. The points are logarithmic means and the limits represent the standard error. The lines were plotted by regression. ■, NZB spleen; □, Ju spleen; ▲, NZB lymph nodes; △, Ju lymph nodes.

To ensure that the difference in response in the spleens and lymph nodes of the two strains of baby mice was not due to a peculiarity in the Ju control strain rather than the NZB mice, baby C57Bl, CBA and Swiss mice were tested at 6, 7 and 8 days after injection. Fig. 3 shows the results for the babies of all strains, plotted as PFC/ $10^6$  viable cells or as PFC per whole spleen or dissected lymph nodes (the same ones in all cases). It can be seen that the organs of the babies of the other control strains gave responses which were similar to the Ju or which differed even more from that of the baby NZB mice.

When the number of viable cells per spleen or in the dissected lymph nodes was plotted

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against time (Fig. 4), the spleens were found to be the same size and to grow at the same rate in baby mice of NZB and Ju strains (the difference in the regression coefficients not being significant), but the lymph nodes were found to grow significantly faster in the baby NZB mice.

Baby mice were injected with SRBC neonatally or when 3 or 5 days old and were tested 6 days after injection. The results (Table 1) suggest that immunological reactivity is not fully developed until the mice are 4–5 days old.

The plaques produced by the baby mice were smaller than those produced by the adults, and the plaques produced by the lymph nodes of the babies were smaller than those produced by the spleen, suggesting that the baby mice produced less antibody per active cell than the adults.

TABLE 1. The immune response of the spleen and the lymph nodes of baby NZB mice challenged with 0.025 ml washed packed SRBC at 0, 3 and 5 days old (they were tested 6 days after injection)

Age when injected (days)	PFC/10 <sup>6</sup> viable cells			
	Spleen		Lymph nodes	
	Individual mice	log mean	Individual mice	log mean
0	0, 1, 1.5, 76	3.3	0, 0, 0, 77	3.0
3	57, 40, 8, 3.5	30	450, 435, 132, 8	120
5	868, 341, 314, 289, 76, 75, 50, 44	155	913, 745, 455, 266, 127, 75	304

## DISCUSSION

Hechtel, Dishon & Braun (1965) have shown that AKR mice do not produce PFC to SRBC when challenged younger than 5 days old, and that Ha/ICR and C57Bl mice do not respond when younger than 7 and 10 days, respectively. They have also shown that the spleen develops competence before the lymph nodes. Their findings are not inconsistent with the results for the Ju baby mice in the present experiments, where the response in lymph nodes and in the spleen was very much reduced and delayed compared with that in the adults. The results obtained with other strains suggest that this is probably a typical immature response of the mouse. The NZB babies show a very different pattern. Even when challenged at 5 days old the spleen gives a response very little different to that of the adult while the lymph nodes give a response which is more sustained than that given by the adult animals. The difference in peak height given by lymph nodes from baby and adult animals was not statistically significant.

Artificial tolerance is produced more easily and with longer lasting effects if the tolerance producing stimulus is given in the neonatal period (see Nossal, 1966) and its maintenance seems to depend upon the persistence of antigen in the animal (Aust, Rogers & Guttman, 1965; Mitchison, 1965). It is tempting to speculate that natural tolerance may also be due to the exposure of the immunological system to small amounts of self-antigens at an age



when it is not capable of responding by antibody formation, thus inducing tolerance which is maintained in later life by the self-antigens present in the system. If this were the case, early maturation or hyperactivity of the immunological system could result in natural tolerance being less firmly established and thus in a tendency to autoimmunity. This explanation is probably too simple and it does not explain why the mice are several months old before showing signs of autoimmune disease, nor why the tolerance should break down only toward specific tissues.

It is likely that the thymus is involved in the development of autoimmunity. In normal mice the thymus is necessary both for the appearance of lymphocytes in the peripheral tissues and for their sensitization to antigen, although it does not appear to be necessary for the proliferation of the cells once they are sensitized (Miller, Mitchell & Weiss, 1967; Zinzar & Svet-Moldavsky, 1967). In NZB mice the thymus shows definite abnormalities. In young mice the reduction of the epithelial component has been reported (de Vries & Hijmans, 1967) and in adult mice 'germinal centres' have been found in the medulla (Abbot & Burnet, 1964; Burnet & Holmes, 1964; Seigler, 1965), although from the present experiments it seems that these do not produce antibody to heterologous antigens. The relationship of the thymus to the development of autoimmunity in NZB mice is not yet clear. It does appear to have a positive effect since grafts of neonatal NZB thymus into the newborn of other strains induce autoimmunity to develop in the recipients later in life, and grafts of neonatal thymus from normal strains into newborn NZB mice do not prevent the appearance of autoimmunity (Howie & Helyer, 1966). Neonatal thymectomy does not prevent the appearance of autoimmunity (Holmes & Burnet, 1966; Howie & Helyer, 1966; East *et al.*, 1967a), showing that the thymus has either affected the immune system before birth or that the thymus is not the only organ involved. Transfer of thymus cells from autoimmune adults to young non-autoimmune NZB mice does not induce autoimmunity although spleen cells similarly transferred will do so (Holmes, 1965), suggesting that the thymus is only effective in the juvenile state or that the mice are only susceptible when very young. A possible explanation is that the thymus induces an abnormal state of immunological responsiveness in the lymphocytes of the embryo and baby NZB mice, perhaps by premature appearance of the humoral factor. This abnormality is revealed a few days after birth by the rapid appearance of immunological responsiveness which may be greater than in the mature normal animal and by the degeneration of epithelial cells in the thymic medulla.

It is impossible to say whether the early competence of the immunological systems of NZB mice is the direct cause of the later development of the autoimmune state or simply another effect of an underlying dysfunction but it seems at least that the basis of the disorder lies in some developmental abnormality of the antibody forming system, whether genetically determined (Bielschowsky & Bielschowsky, 1964; Burnet & Holmes, 1965) or induced by the virus particles found in axenic NZB mice (East *et al.*, 1967b; Mellors & Huang, 1967).

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THE EFFECT OF HYPOPHYSECTOMY ON THE IMMUNE RESPONSE

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Short title : Hypophysectomy and the immune response

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### SUMMARY

Twenty one day old rats were hypophysectomised or sham operated and challenged with sheep red blood cells 5 or 21 days after operation. Although the sizes of the bodies and lymphoid organs were greatly decreased in the hypophysectomised animals no significant difference was found in the numbers of plaque forming cells or serum antibody titres against the sheep red blood cells. The histology of the hypophysectomised rats showed a cell depletion in the perifollicular zone of the spleen follicles, but no obvious histological differences in the thymus.

It is concluded that pituitary hormones are not directly necessary for the formation of humoral antibodies.

### INTRODUCTION

Observations in experimental animals of the effects of treatment with different hormones on the thymus and peripheral lymphatic tissue suggests that a critical hormone balance regulates the growth and development of these organs (Ernström, 1965; Branceni, 1968; Dougherty, Berliner, Schneebeli and Berliner, 1964; Bearn, 1968). The involutionary effect of corticosteroids and gonadal hormones (Branceni, 1968; Dougherty *et al.*, 1964; Warner and Burnet, 1961; Lundin, 1958) seems to be antagonised by growth promoting and developmental hormones such as growth hormone and thyroxin (Ernström, 1965; Lundin, 1958).

More recently, Pierpaoli and Sorkin (1967a) demonstrated changes in the hypophysis in neonatally thymectomised mice suggesting a functional relationship between the hypophysis and the thymus. Other experiments using mice treated with antiserum to pituitary hormones (Pierpaoli and Sorkin, 1967b, 1968, 1969 a, b) or using a hypopituitary dwarf strain of mice (Baroni, 1967; Baroni, Fabris and Bertoli, 1967, Pierpaoli, Baroni, Fabris and Sorkin, 1969) suggests a relationship between immune function and the pituitary.



It was decided to elucidate further the role of pituitary hormones by testing the effect of the removal of the pituitary on the immune response to sheep red blood cells (SRBC).

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley strain rats hypophysectomised or sham hypophysectomised at the age of 21 days were obtained from the Charles River Breeding Laboratories Inc., Boston, Mass., U.S.A. When the animals were killed the completeness of hypophysectomy was determined by macroscopic observations of the pituitary fossa, by palpation of the gonads and by regression in the weight of the adrenals and the whole body. Animals with pituitary remnants, unregressed gonads, or high body or adrenal weight were rejected.

### Antigen Challenge

Rats were injected intraperitoneally with 0.1 ml. washed packed sheep erythrocytes (SRBC) ( $2 \times 10^9$ ) 5 days after hypophysectomy or sham operation (26 days old). In a later experiment the animals were injected with the same quantity of SRBC at 21 days after hypophysectomy (42 days old) and for this experiment the controls were unoperated Sprague-Dawley rats of the same age.

### Detection of immune response

The rats were killed 4, 7 and 10 days after injection in the case of the rats injected 5 days after operation, and 6 days after injection in the case of the rats injected 21 days after operation. The rats were weighed and blood taken for assay of serum haemolysin and haemagglutinin titres. The serum was stored at  $-20^{\circ}\text{C}$  until the experiments were complete, and estimations made at the same time on all the sera. Spleens, thymuses and adrenal glands were removed and weighed. Twenty thymuses and 4 spleens of hypophysectomised and 12 thymuses and 4 spleens of sham operated animals

were fixed in Carnoy solution overnight. The sections were stained with Haematoxylin and Eosin. The remainder of the spleens were assayed for haemolysin plaque forming cells (PFC) by the method of Jerne, Nordin and Henry (1963) as modified by Evans, Williamson and Irvine (1968).

#### Serum protein

Standard methods (Ouchterlony, 1967) were used for immunoelectrophoresis. The antiserum used was a polyvalent goat anti-rat-gamma-globulin serum (Highland Division, Travenol Lab. Inc., Los Angeles, California). The slides were washed and dried and stained with Amido Black. Quantitative cellulose acetate electrophoresis was kindly performed by the Chemistry Department, Royal Infirmary, Edinburgh, by standard methods.

#### Statistical analysis

Geometric means and standard errors of all parameters were determined, and the significance of the differences calculated by Student 't' test.  $P > 0.05$  were classed as not significant.

### RESULTS

#### The effect of hypophysectomy on body and adrenal weight

The effectiveness of hypophysectomy was clearly evident when the weights of the body and lymphoid organs (Fig. 1) and of the adrenals (Table 1) were compared in hypophysectomised and sham operated animals. Fig. 1a shows the weights of the rats at 4, 7 and 10 days after injection, that is 30, 33 and 36 days of age or 9, 12 and 15 days after operation. The weights of the sham operated animals continued to increase, while that of the hypophysectomised animals began to decrease between 12 and 15 days after operation. The same trends were seen for the adrenal weight. The differences between both the mean body and adrenal weights of the sham operated and hypophysectomised animals were significant at the  $p < 0.001$  level, at the three time intervals.

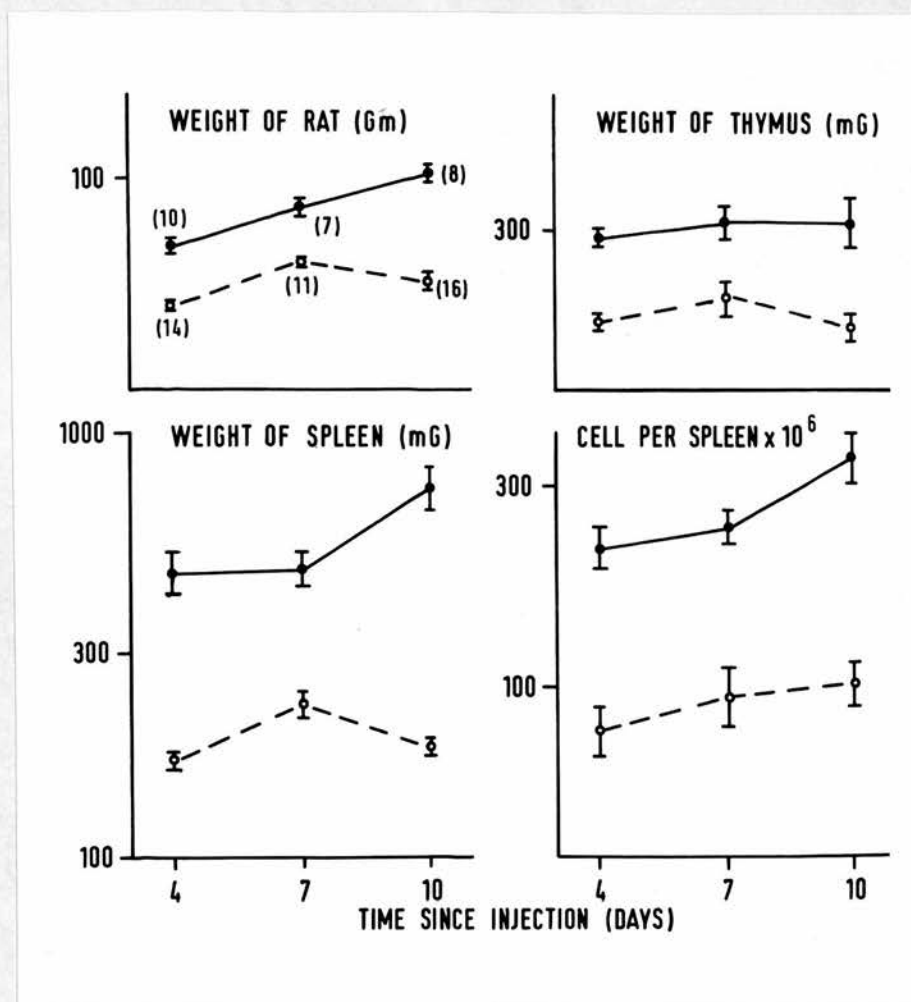


Fig. 1

The sizes of the body and the lymphoid organs of hypophysectomised (0 -- 0) and sham operated (0 — 0) rats. (a) Body weight; (b) thymus weight; (c) spleen weight; (d) number of viable spleen cells  $\times 10^6$ . The rats were operated on at 21 days of age, challenged with sheep red cells 5 days after operation and measured 4, 7 and 10 days after antigen challenge. The points are geometric means, the limits are standard errors and the number of animals in each group is indicated in parenthesis on graph (a).

TABLE I

	Time since operation (days)		
	9	12	15
No. of hypophysectomised rats	14	11	16
Adrenal weight (mg.)	10.0	9.3	7.2
S.E.	10.5 - 9.5	9.8 - 8.7	7.8 - 6.3
No. of sham operated rats	8	7	8
Adrenal weight (mg.)	21.4	20.0	25.7
S.E.	23.5 - 19.0	23.4 - 17.8	16.9 - 24.0
P	0.001	0.001	0.001

The weights of the adrenals of rats hypophysectomised or sham operated at 21 days of age. The table shows the geometric means, the limits of the standard error and the significance of the differences between the hypophysectomised and control groups.



### The effect of hypophysectomy on the structure of the lymphoid organs

Fig. 1b and Fig. 1c show the weights of the thymuses and the spleens of the same rats. Obvious differences were seen between the hypophysectomised and the sham operated animals and these differences were significant at the  $p < 0.001$  level (except in the case of the thymus weight 7 days after injection, where the difference was only significant at the 0.01 level). Again the weights of the lymphoid organs of the sham operated animals increased over the period of the experiment, while those of the hypophysectomised animals began to decrease between 12 and 15 days after operation. This difference was particularly marked in the spleen. Furthermore, in proportion to the total body weight, the spleens in the hypophysectomised animals were significantly smaller than in the sham operated controls ( $p < 0.001$ ). Although the thymuses were also proportionately smaller in the hypophysectomised animals the difference compared to the controls did not reach statistical significance.

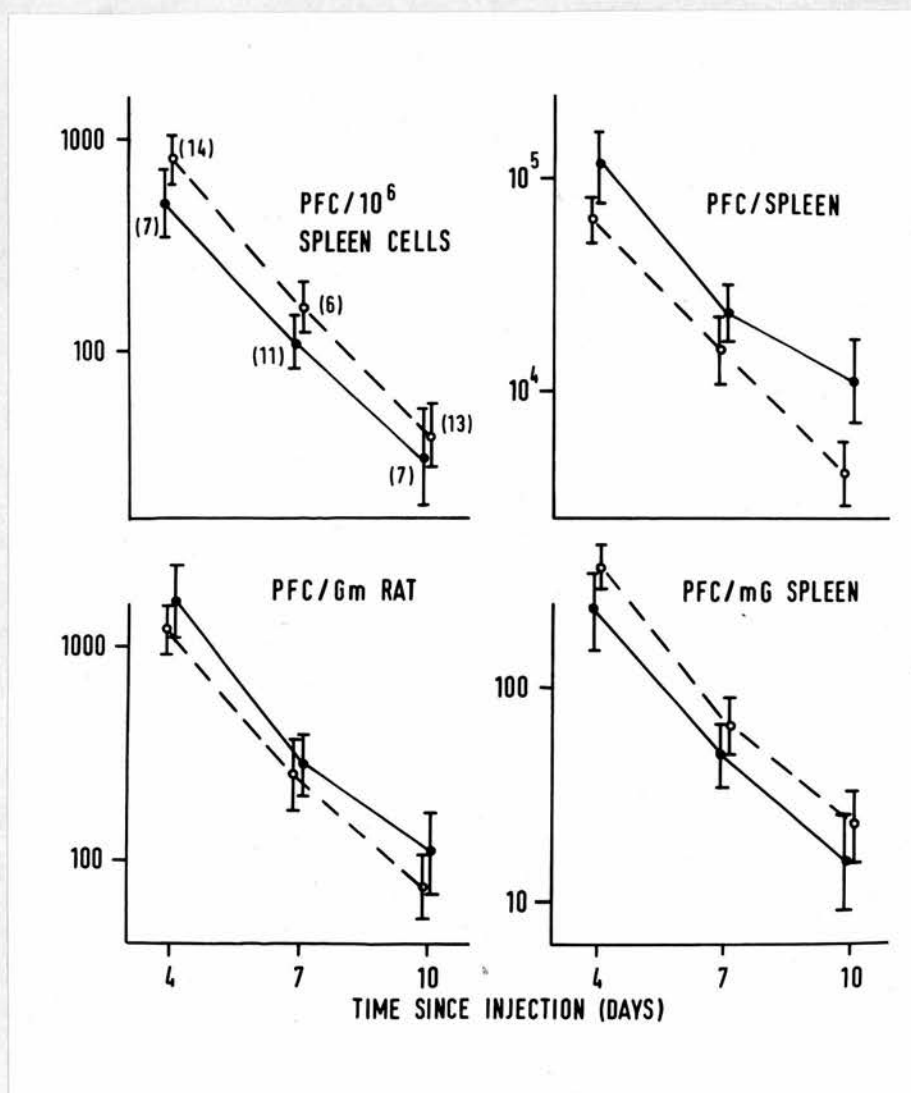
The results for the viable cell count per spleen were similar to those obtained by measuring the weight of the spleen. The differences between the hypophysectomised and sham operated animals were again significant at the  $p < 0.001$  level.

### Histology

In the spleen the outstanding feature was an atrophy of the follicles with a cell depletion in the perifollicular zone. No obvious histological changes were found in the thymus.

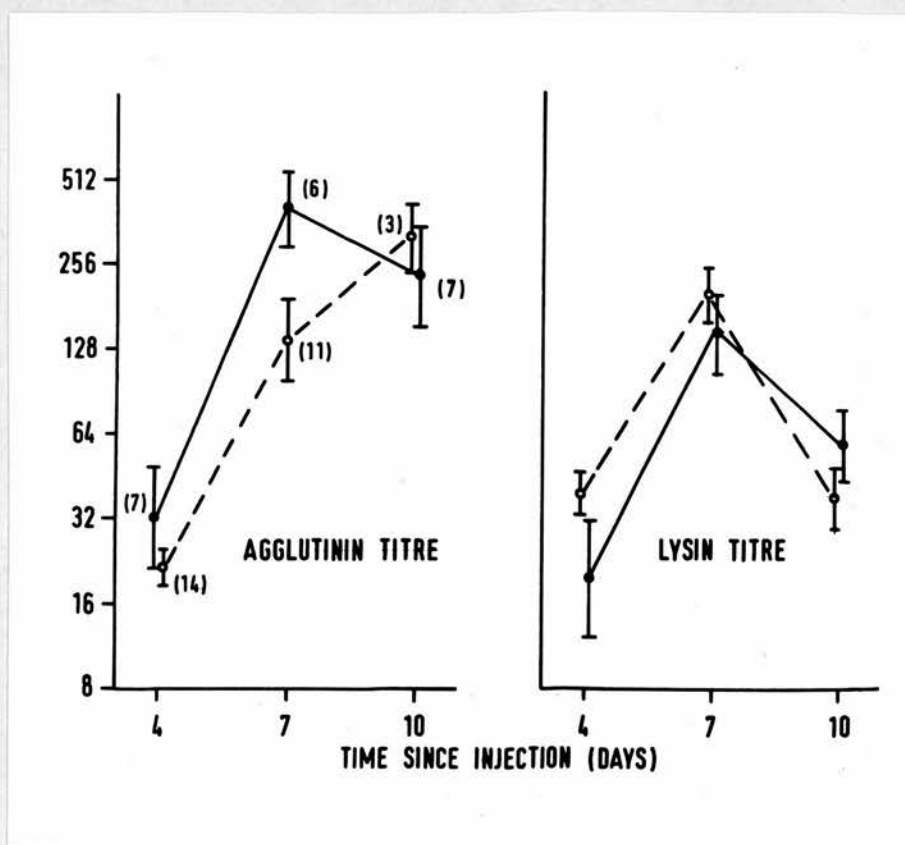
### The effect of hypophysectomy on the function of the lymphoid organs

Fig. 2 shows the results when the spleens of the hypophysectomised and sham operated animals were assayed for plaque forming cells. At no time and by none of the methods of calculation were the results for the hypophysectomised and sham operated rats significantly different from one another.



**Fig. 2**

Plaque forming cells in hypophysectomised (0 -- 0) and sham operated (0 — 0) rats. (a) PFC per  $10^6$  viable spleen cells, (b) PFC per whole spleen, (c) PFC per gm. body weight, (d) PFC per mg. spleen weight. The rats were operated at 21 days of age, challenged with sheep red cells 5 days after operation and measured 4, 7 and 10 days after antigen challenge. The points are geometric means, the limits are standard errors and the number of animals in each group is indicated in parenthesis on graph (a).



**Fig. 3**

Serum antibody response of hypophysectomised (0 -- 0) and sham operated (0 — 0) rats. (a) Reciprocal haemagglutinin titres, (b) reciprocal haemolysin titres. The rats were operated at 21 days of age, challenged with sheep red cells 5 days after operation and measured 4, 7 and 10 days after antigen challenge. The points are geometric means, the limits are standard errors and the number of animals in each group is indicated in parenthesis on graph (a).

TABLE 2

	No. of animals	PFC/ $10^6$	PFC/whole spleen/ $10^4$	PFC/gm rat weight	PFC/mg spleen weight	Rat weight (gm.)	Spleen weight (mg.)	Cells per spleen/ $10^6$
Hypophysectomised	6	45.6	2.88	426	155	67.5	186	616
S.E.		61.6-33.9	4.36-1.91	615-288	214-115	70.8-66.0	209-170	760-490
Control	8	26.3	3.72	182	50.0	145	550	1,490
S.E.		38.9-17.4	5.50-2.57	256-138	66.0-37.2	151-141	562-525	1,620-1,320
p		N.S.	N.S.	N.S.	<0.05	<0.001	<0.001	<0.01

The immune response and the body and spleen size of hypophysectomised and control rats. The rats were hypophysectomised at 21 days of age and injected with SRBC at 21 days later. Controls were unoperated rats of the same age. The table shows geometric means, the limits of the standard error and the significance of the differences between the hypophysectomised and control groups.



As shown in Fig. 3 there was no significant difference in the haemolysin titres in the hypophysectomised versus sham operated controls following challenge with sheep red cells (day 4 after injection  $p > 0.1$ , day 7  $p > 0.4$ , day 10  $p > 0.2$ ). The haemagglutinin titres in the sham operated and hypophysectomised animals on days 4 and 10 were not significantly different ( $p < 0.2$ ,  $p < 0.5$  respectively) but on day 7 there was a difference which just reached significance ( $p < 0.05$ ). Taking the other results into consideration it is doubtful that this indicates an important difference between the hypophysectomised and sham operated animals.

In case the five days that we had allowed to elapse between hypophysectomy to become fully apparent (although considering the effects on the weight of the animals and the weight of the lymphoid organs, this does not seem likely), six animals were injected at 21 days after hypophysectomy and tested six days after injection. The controls for this group were eight unoperated Sprague-Dawley rats of the same age. Once again the weights of the bodies and of the spleens were significantly lower in the hypophysectomised animals, while the antibody response was not (Table 2).

#### Serum protein

Quantitative electrophoresis revealed no significant differences in the content of any of the serum fractions between the hypophysectomised and control animals. The sera from all the animals challenged with antigen five days after hypophysectomy was subjected to immunoelectrophoresis using a polyvalent anti-rat-gammaglobulin serum. No differences in the gammaglobulins of the hypophysectomised and control animals were observed.

### DISCUSSION

From the marked changes in the adrenal and total body weights and from autopsy inspection of the pituitary fossa, it is clear that hypophysectomy was effective. Even if microscopic amounts of pituitary tissue had remained,

Lundin (1958) has shown histologically that such animals show no differences in the lymphatic tissue compared to totally hypophysectomised animals.

Our experiments show that there was no significant difference in the immune response of Sprague-Dawley rats hypophysectomised at 21 days of age and challenged with sheep red blood cells 5 or 21 days later, when compared to controls. The half lives in the plasma of growth hormone (GH) (Cornblable *et al.*, 1965) and thyroid stimulating hormone (TSH) (Kirkham, 1966) are 20-30 minutes although the biological activity may last somewhat longer. Therefore pituitary hormones are unlikely to be directly necessary for the immune response.

Since the spleens of the hypophysectomised animals are significantly smaller than the spleens of the sham operated controls, it would be expected either that the reduction has occurred in antibody-forming cells to an equal degree with the non-antibody-forming cells in which case total PFC would be reduced, or that the reduction has been only in the non-antibody-forming cells in which case the proportion of PFC would be increased in the hypophysectomised animals. Since the results suggest that both these have occurred to a slight degree it is concluded that while reduction in size and cellularity of the spleen involves some of the antibody-forming elements it involves a larger proportion of non-antibody-forming cells.

The spleens of the hypophysectomised animals showed an atrophy of the lymph follicles with a loss of the parafollicular area which is in agreement with other observations in hypophysectomised animals (Lundin, 1958; Ernström, 1965). However, as Osoba and Miller, (1964) originally showed in neonatally thymectomised animals, cellular depletion of the spleen does not result in reduction of the immune response provided the thymic humoral factor is present. The cellular depletion of the lymphoid tissues observed in hypophysectomised animals may be part of a generalised phenomenon since it has been shown by Crean (1968) that hypophysectomy in 4-6 week old rats

causes a reduction in the stomach weight and in the mass of the gastric mucosa with a proportional decrease in the number of the parietal cells.

It has been suggested (Baroni et al., 1967; Pierpaoli et al., 1969; Pierpaoli and Sorkin, 1968, 1969a, b) that thyroxin and/or growth hormone may play a role in the immune response. This hypothesis is based on two main lines of experimentation. The first involves the hereditary pituitary dwarf Snell-Bagg mouse. These animals, when compared to normal litter mate controls, show an extreme reduction in the ability to produce PFC to sheep red cells (Baroni, 1967; Baroni et al., 1967; Pierpaoli et al., 1969). The response could be restored to normal by treatment with growth hormone or thyroxin, or most successfully with both together (Pierpaoli et al., 1969). The other line of experimentation involves the treatment of mice with anti-pituitary, anti-GH or anti-TSH sera. This resulted in a wasting syndrome in most of the mice treated at 15-30 days old, similar to that seen in neonatally thymectomised mice (Pierpaoli and Sorkin, 1967b, 1968, 1969a, b). Although our animals virtually stopped growing they did not show wasting. In addition, Pierpaoli et al., (1969) and Pierpaoli and Sorkin (1969a) have reported an absence of production of precipitating antibody to rabbit gamma-globulin in young male Charles River mice of 5-8 weeks of age treated with anti-GH or anti-TSH sera. These responses could be restored to normal by treatment with either GH or TSH.

The most probable explanation, and one which Pierpaoli et al. (1969) have also suggested, is that the hormones act in the development of immune responsiveness (perhaps by influencing the thymic control of the maturation of the peripheral antibody-forming organs) and that once the animals are immunologically mature, the action of the hormones is no longer necessary. Indeed, Pierpaoli et al. (1969) have mentioned preliminary experiments in the dwarf mouse strain, indicating that after 20 days of hormone treatment, reconstituted dwarf mice did not lose their antibody-forming capacity for at least 30 days. This is analogous to the case for thymectomy.

This hypothesis could explain the results with the hypopituitary dwarf mice, but not the results with anti-GH and anti-TSH treatment. In the latter experiments some of the mice were treated at an age which was comparable to that when our rats were hypophysectomised. Therefore, either the anti-GH and anti-TSH sera worked by cross-reaction with an antigen in some other tissue (perhaps a minor but important component of lymphoid tissue) or, alternatively, strain differences between their mice and our rats may be important. The results with the dwarf mice could be due to a pleiotropic effect of the dw gene and the reconstitution of the immune response with the pituitary hormones due to a non-specific growth or metabolism-promoting effect.

#### ACKNOWLEDGEMENTS

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The effect of *Bordetella Pertussis* vaccine on the development of  
experimental thyroiditis in rats immunized by the intra-lymph  
node route or into a hind footpad

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SHORT COMMUNICATION

THE EFFECT OF *BORDETELLA PERTUSSIS*  
VACCINE ON THE DEVELOPMENT OF  
EXPERIMENTAL THYROIDITIS IN RATS IMMUNIZED  
BY THE INTRA-LYMPH NODE ROUTE OR INTO A  
HIND FOOTPAD

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SUMMARY

Experimental thyroiditis was produced by a single injection of homologous thyroglobulin in Freund's complete adjuvant when this was given along with an intracutaneous injection of *Bordetella pertussis* vaccine. Severe thyroid lesions were observed as early as 8 days. There was no significant difference in the incidence and severity of thyroiditis and antibody formation when the thyroglobulin was injected into lymph nodes or into a hind footpad.

INTRODUCTION

Pertussis vaccine has been shown to have an enhancing effect on the development of experimental autoallergic diseases in laboratory animals (Lee & Olitsky, 1955; Levine & Wenk, 1967, 1968; Hargio, Malkiel & Berkelhammer, 1968; Paterson & Drobish, 1968). The purpose of the present paper is to report further studies in relation to experimental thyroiditis in rats.

MATERIALS AND METHODS

*Animals*

Female inbred Wistar rats 4–6 weeks old were obtained from animal suppliers (London) Ltd, and used for immunization.

*Immunization schedule*

According to the injection route animals were immunized either with 0.1 ml of an emulsion containing 2 mg rat thyroglobulin in Freund's complete adjuvant (CFA) into a

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hind footpad or by the injection of 0.01 ml emulsion containing 0.2 mg of rat thyroglobulin in CFA in each of two cervical lymph nodes using a 28-gauge needle and a tuberculin syringe. Control animals received the same volumes of an emulsion of saline and CFA. The animals were killed 8, 14 and 17 days after the immunization. Half the animals immunized into a hind footpad were injected with  $2 \times 10^9$  *Bordetella pertussis* organisms intradermally into the dorsum of the same foot used for antigen injection. Half the rats immunized into two cervical lymph nodes were also injected with  $2 \times 10^9$  *B. pertussis* organisms intradermally into the inner site of the neck skin. The pertussis vaccine injections were always made immediately after the antigen administration. A concentrate of pertussis vaccine containing  $80 \times 10^9$  organisms/ml was generously supplied by Wellcome Research Laboratories, Langley Court, Beckenham, England.

#### *Circulating antibodies*

Immunofluorescence and tanned red cell tests were employed for the detection of thyroid circulating antibodies as previously described (Kalden *et al.*, 1968).

#### *Histology*

The rat thyroids were fixed in formol saline (4%) and stained with haematoxylin and eosin (H & E). The degree of thyroiditis was scored as follows: — = normal thyroid structure, + = focal thyroiditis, ++ = more severe thyroiditis, +++ = diffuse thyroiditis with altered thyroid structure, and ++++ = severe thyroiditis with goitre. The arithmetic mean score of the thyroid lesions is given for each group.

## RESULTS

#### *Histology*

As shown in Table 1 the injection of *B. pertussis* vaccine immediately after the antigen injection increased significantly ( $P < 0.001$ ) the incidence and severity of the thyroid lesions. Regardless of the route of antigen injection severe thyroiditis was found as early as 8 days after immunization provided *B. pertussis* was used (mean scores 2.3 and 3 for immunization into lymph nodes or into a hind footpad, respectively) (Fig. 1). The highest mean score 3.2 of thyroid lesions was found 17 days after immunization in rats immunized into a hind foot. There was no significant difference in the grade of thyroiditis in rats immunized by the intra-lymph node injection route and rats immunized in a hind footpad with or without the additional use of *B. pertussis* vaccine.

When *B. pertussis* vaccine was not used as an additional adjuvant only mild thyroiditis was produced in rats immunized via the cervical lymph nodes or hind footpad.

#### *Circulating antibodies*

Rats immunized with thyroglobulin and CFA produced higher ( $P < 0.02$ ) thyroglobulin antibody titres when *B. pertussis* vaccine was used as additional adjuvant. Production of thyroglobulin antibodies was apparent by 8 days.

The finding with the indirect immunofluorescence test using fixed rat thyroid sections confirmed the results obtained by the tanned red cell test. Immunofluorescence using unfixed thyroid sections did not detect any antibody against rat thyroid cytoplasm.

TABLE 1. The effect of *B. pertussis* vaccine on the development of experimental thyroiditis and circulating antibody response in rats immunized with rat thyroglobulin into two cervical lymph nodes or a hind footpad

Substance injected in CFA	Site of injections	Pertussis vaccine	No. of rats*	Mean score of thyroiditis	Significance	Mean antibody titres†	Significance
Rat thyroglobulin	Cervical nodes	No	6	1.5	$P < 0.001$	2	$P < 0.02$
Rat thyroglobulin	Cervical nodes	Yes	9	2.8		4	
Saline	Cervical nodes	Yes	6	0.3		0	
Rat thyroglobulin	Footpad	No	12	0.75	$P < 0.001$	2.5	$P < 0.02$
Rat thyroglobulin	Footpad	Yes	12	2.9		4.25	
Saline	Footpad	Yes	4	0		0	

CFA, Freund's complete adjuvant.

\* An equal number of rats in each group was studied at 8, 14 and 17 days after immunization.

† Mean log base 2 titres.

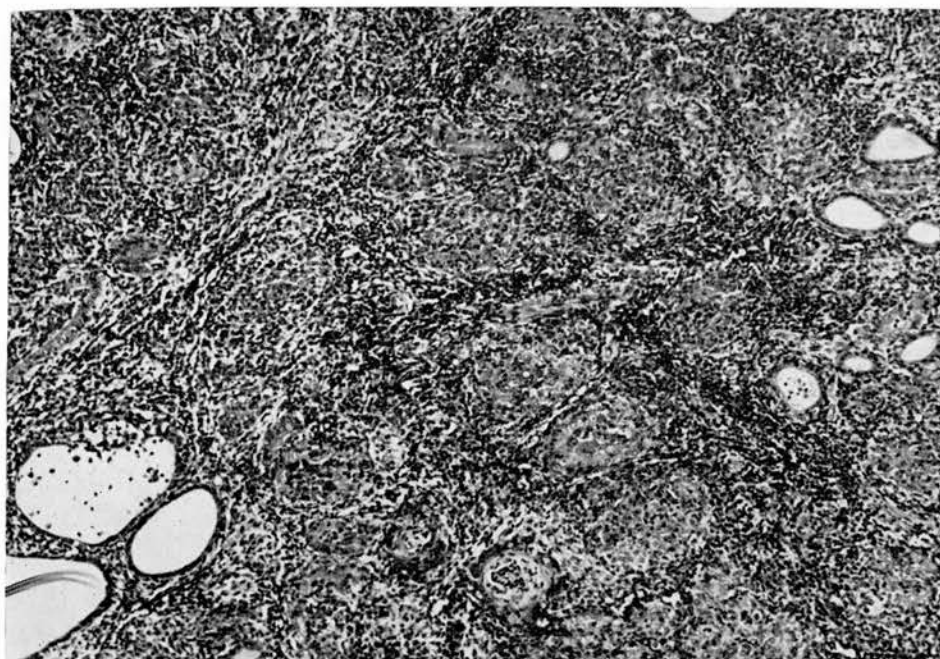


FIG. 1. Thyroid of a rat 8 days after the immunization with 2 mg rat thyroglobulin in Freund's complete adjuvant into a hind footpad followed immediately by the intradermal injection of  $2 \times 10^9$  *B. pertussis* organisms. Note the severe inflammatory reaction (grade 4) with involvement and destruction of the follicles. H & E,  $\times 96$ .

### DISCUSSION

The observation of a more rapid and more severe production of experimental thyroiditis in rats when *B. pertussis* vaccine is given along with the antigen injection is in keeping with the findings reported briefly by Paterson & Drobish (1968) who used a crude thyroid extract in CFA for immunization. The present paper indicates that moderate to severe thyroiditis may be established within 8 days with the production of circulating thyroglobulin antibody but not thyroid complement fixing antibody.

In agreement with Horne & White (1968) and in contrast to Newbold (1964, 1965), we could not show any superiority of the intra-lymph node injection route over the usual footpad injection in terms of induction of cell mediated hypersensitivity and circulating antibody response. One advantage of the intra-lymph node route is the smaller amount of antigen needed for the production of specific organ lesions and circulating antibody.

### ACKNOWLEDGMENTS

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The suppression of experimental thyroiditis in the rat by  
heterologous anti-lymphocyte globulin

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## THE SUPPRESSION OF EXPERIMENTAL THYROIDITIS IN THE RAT BY HETEROLOGOUS ANTI-LYMPHOCYTE GLOBULIN

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(Received 28 May 1968)

### SUMMARY

The auto-allergic thyroiditis produced in rats by the injection of rat thyroglobulin in complete Freund's adjuvant can be suppressed by the administration of the IgG immunoglobulin fraction of heterologous (horse) anti-lymphocytic serum (ALS-IgG). ALS-IgG also suppressed the formation of circulating antibodies against rat thyroglobulin.

### INTRODUCTION

The ability of anti-lymphocytic serum to suppress a variety of cell mediated and humoral immune responses is well documented and has been recently reviewed (James, 1967, 1968). In particular it has been demonstrated that anti-lymphocytic serum suppresses the development of autoallergic encephalomyelitis (Waksman, Arbouys & Arnason, 1961; Leibowitz, Lessop & Kennedy, 1968), of Freund adjuvant arthritis (Currey & Ziff, 1966, 1968) and of haemolytic anaemia in NZB mice (Denman, Denman & Holborow, 1967). The present paper describes the effect of anti-lymphocytic serum on the development of experimental thyroiditis in rats.

### MATERIALS AND METHODS

#### *Animals*

Two- to 3-month-old male rats derived from a random bred Wistar strain maintained in the breeding station of the University of Edinburgh were used for immunization.

#### *Preparation of thyroglobulin*

Rat thyroids (stored at  $-20^{\circ}\text{C}$  prior to use) were minced and then disrupted in 2 volumes of phosphate-buffered saline, pH 7.2, in a ground glass homogenizer. The resultant suspension was centrifuged at  $4^{\circ}\text{C}$  for 10 min at 30,000 g and the crude thyroglobulin was precipitated from the supernatant with ammonium sulphate (saturation between 40 and 42%). The precipitate was dialysed and freeze dried.

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*Preparation of anti-rat lymphocyte serum and normal horse IgG*

The ALS was produced in a horse by the intravenous injection of thoracic duct lymphocytes obtained following the cannulation of random bred Wistar rats. The horse received six injections at weekly intervals followed by a seventh injection after a further 2 weeks. Each injection consisted of  $1.2-1.5 \times 10^9$  cells of which 87-95% were viable. Two weeks after the last injection, the animal was bled and the serum obtained was inactivated by incubation at 56°C for 30 min.

Immunoglobulin G fractions were prepared from this antiserum and from normal horse serum by a combined salt precipitation and chromatographic procedure. The initial step involved the precipitation of a crude globulin concentrate by the addition of 1 volume of 28% (w/v) sodium sulphate. The precipitate was re-dissolved in 0.15 M-sodium chloride and re-precipitated as above. This material was then dissolved in and dialysed against 0.02 M-phosphate buffer, pH 6.5, and then batched as Whatman DE11 diethyl-aminoethyl cellulose exchanger with an ion exchange capacity of 1.0 mEq/g. The final products were concentrated by lyophilization and reconstituted when required in 0.06 M-phosphate buffered saline, pH 7.2, containing 0.15 M-sodium chloride. The 1 g/100 ml anti-lymphocytic IgG solution so obtained was then absorbed with one-quarter its volume of rat erythrocytes to remove erythrocyte agglutinins. Both preparations of the ALS-IgG and normal horse IgG (NH-IgG) were finally sterilized by filtration through an 0.22  $\mu$ m millipore filter. Immuno-electrophoretic analysis using a rabbit anti-horse serum indicated that the products contained several IgG components. The reciprocal lympho-agglutination and lymphocytotoxic titres of the anti-rat lymphocyte IgG were both 256 while the normal horse IgG possessed negligible amounts of anti-lymphocyte activity (Abaza & Woodruff, 1966).

*Immunization schedule*

Each of the thirty rats was injected intracutaneously into the base of the tail with 0.05 ml emulsion containing 2 mg of the rat thyroglobulin preparation in complete Freund's adjuvant (CFA). The injection was repeated 21 days later. Ten control rats were immunized with 0.05 ml saline in CFA and injections were also repeated 21 days later. The animals were killed after 4 weeks and serum stored at -20°C.

*Treatment schedule*

The thirty test animals were divided into three equal groups. Taking the time of the first sensitization to rat thyroglobulin as day 0, the first group of ten animals was injected with 3 ml of 1 g/100 ml ALS-IgG solution intraperitoneally on days -3, -2 and -1. From day 2 on and on every 2nd day thereafter, these animals were given 2 ml ALS-IgG solution intraperitoneally. The second group of test animals (ten animals) were treated with 1 g/100 ml NH-IgG solution following the same schedule. The third group of ten test animals received no treatment. One of these animals died. A further group of ten control rats received saline in CFA but were not treated with ALS-IgG or NH-IgG (Table 1).

*Detection of circulating antibody*

Tanned sheep red cells were sensitized with one of the following antigens: (1) 2 mg/ml rat thyroglobulin, (2) 5 mg/ml solution of ALS-IgG, or (3) 5 mg/ml solution of NH-IgG, and incubated overnight at room temperature with doubling dilutions of the sera from the test and control animals (Herbert, 1968).



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The indirect Coombs test was also employed to detect antibody to thyroglobulin. Goat anti-rat  $\gamma$ -globulin (Hyland Division, Travenol Laboratories Inc., Los Angeles) was conjugated with fluorescein isothiocyanate and incubated with cryostat sections of rat thyroid 4–6  $\mu$  thick and fixed in methanol at 56°C for 3 min. In order to test for complement fixation, fixed thyroid sections were incubated with inactivated test sera and guinea-pig complement and then with anti-guinea-pig complement serum conjugated with fluorescein isothiocyanate. Both of these immunofluorescence methods were also employed to unfixed sections of rat thyroid to detect thyrocytoplasmic antibody in the test sera.

*Histology*

The thyroid gland, thymus, lymph nodes and spleen were fixed overnight in Carnoy solution and the sections were stained by haematoxylin and eosin (H & E), methyl-green-pyronine, and PAS. The kidneys were fixed in formol saline (4%) and stained with H & E alone.

*Scoring of thyroiditis*

The degree of thyroiditis was scored as follows:

- = Normal thyroid structure.
- + = Focal thyroiditis.
- + + = More severe thyroiditis.
- + + + = Diffuse thyroiditis with altered thyroid structure.
- + + + + = Severe thyroiditis with goitre.

The arithmetic mean score for each group of animals was obtained by totalling the number of ‘+’s for the thyroid lesions and dividing by the number of animals. The thyroid sections were randomized before they were read.

Absolute lymphocyte counts were obtained from total white cell haemocytometer counts and differential counts on Pappenheim stained blood smears and are expressed as counts per cubic millimeter blood.

**RESULTS**

Treatment with ALS-IgG prevented the development of thyroiditis in response to sensitization with thyroglobulin in CFA. Rats sensitized with rat-thyroglobulin in CFA and treated with saline developed histological evidence of thyroiditis with the mean score of 1.4, and a similar degree was seen in thyroglobulin immunized rats treated with NH-IgG (mean score 1.0) (Fig. 1). In contrast, the thyroid of animals treated with ALS-IgG closely resembled those of animals given only saline in CFA (Table 1).

Furthermore, ALS-IgG also prevented the development of antibody to thyroglobulin in rats subsequently sensitized with rat thyroglobulin in CFA. NH-IgG had no such effect (Table 2). The tanned red cell titres in the animals sensitized with rat thyroglobulin CFA that were not given any heterologous globulin ranged up to 1:1280 and, in those animals given NH-IgG, the titres ranged up to 1:640. None of the tanned red cell titres was positive in those immunized rats that received ALS-IgG treatment.

The findings with the indirect immunofluorescence test using fixed rat thyroid sections

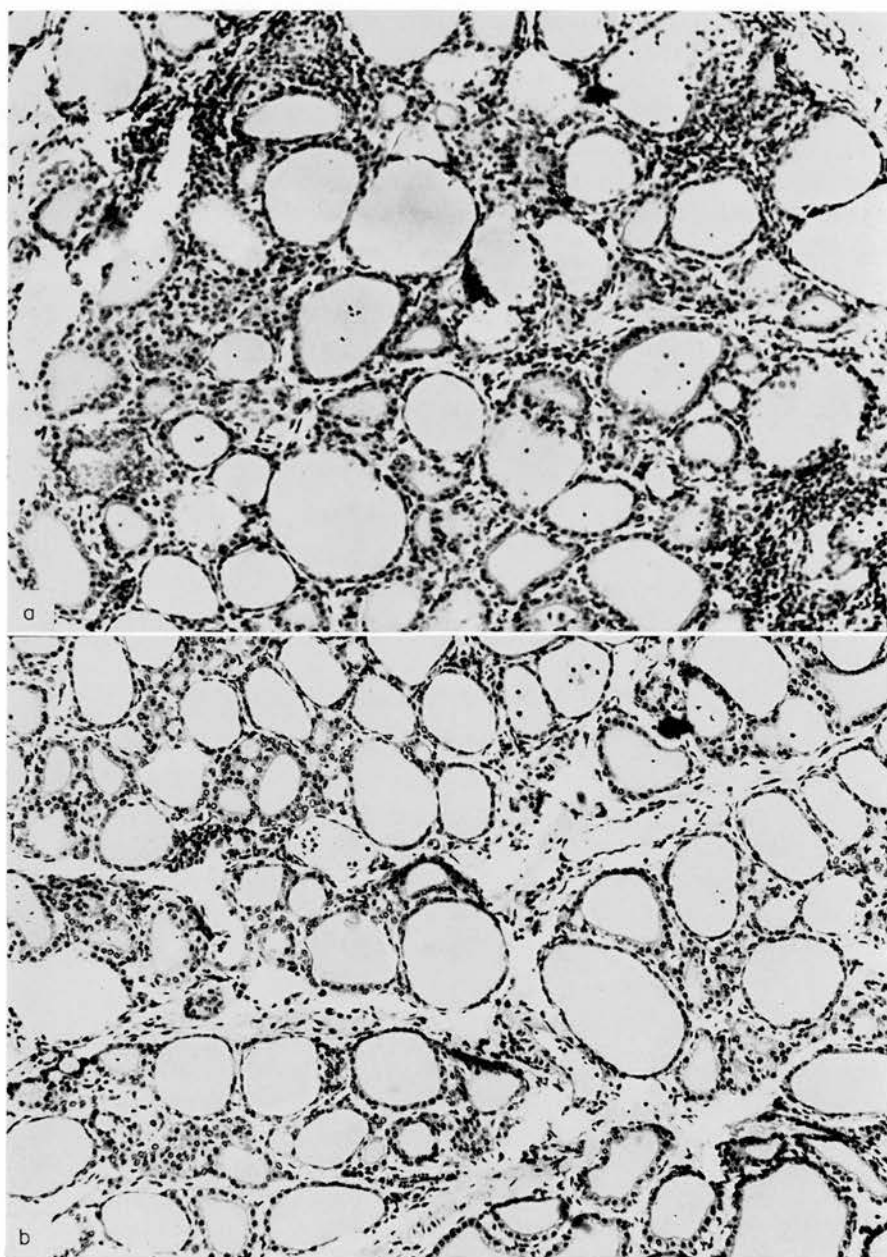


FIG. 1. (a) Thyroid of a rat treated with normal horse IgG and subsequently sensitized to rat thyroglobulin in CFA. Note the inflammatory reaction with involvement and destruction of some follicles. H & E,  $\times 85$ . (b) Thyroid of a rat treated with horse anti-rat lymphocytic IgG and subsequently sensitized to rat thyroglobulin in CFA. The thyroid shows an intact structure. H & E,  $\times 85$ .

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confirmed the results of the tanned cell method insofar that staining of the colloid was not seen in the animals treated with ALS-IgG, except in one doubtful case.

Immunofluorescence using unfixed thyroid sections did not detect any antibody against rat thyroid cytoplasm whether or not the sensitized animals had been treated with ALS-IgG.

TABLE 1. The effect of anti-rat-lymphocytic serum (ALS-IgG) on the production of experimental thyroiditis in the rat by sensitization with rat thyroglobulin in complete Freund's adjuvant

Sensitization	Treatment	No. of rats	Severity of thyroiditis*					Mean† score
			—	+	++	+++	++++	
Rat thyroglobulin + CFA	Saline	9	3	2	2	1	1	1.4
Rat thyroglobulin + CFA	NH-IgG‡	10	4	3	2	1	0	1.0
Rat thyroglobulin + CFA	ALS-IgG§	10	9	1	0	0	0	0.1
Saline + CFA	None	10	10	0	0	0	0	0

\* Gradation of thyroid lesions as described under 'Materials and methods'.

† Lesions were scored as follows: — = 0; + = 1; ++ = 2; +++ = 3; ++++ = 4.

‡ Normal horse IgG.

§ Anti-rat-lymphocytic serum.

Likewise, indirect immunofluorescence methods did not demonstrate any fixation of complement in the control and sensitized animals either with regard to the colloid or to the thyroid cytoplasm.

Fig. 2 shows the fall in the absolute peripheral lymphocyte count in animals treated with ALS-IgG in contrast to the animals given no treatment or only normal horse serum. Four

TABLE 2. The effect of ALS-IgG on the production of antibody to thyroglobulin in rats subsequently sensitized with rat thyroglobulin in CFA

No. of rats	Sensitization	Treatment	Thyroglobulin antibody	
			Tanned cell titre $\geq 1/20$	Immunofluorescent
9	Rat thyroglobulin + CFA	None	6	8
10	Rat thyroglobulin + CFA	NH-IgG	5	6
10	Rat thyroglobulin + CFA	ALS-IgG	0	(1)
10	Saline + CFA	None	0	0

hours after a single injection of ALS-IgG, there was a significant fall in the peripheral lymphocyte count and this depression was maintained with continuous treatment for approximately 3 weeks after which it rose to almost normal levels in spite of continuing ALS-IgG injection. A slight depression was initially observed in the control groups.

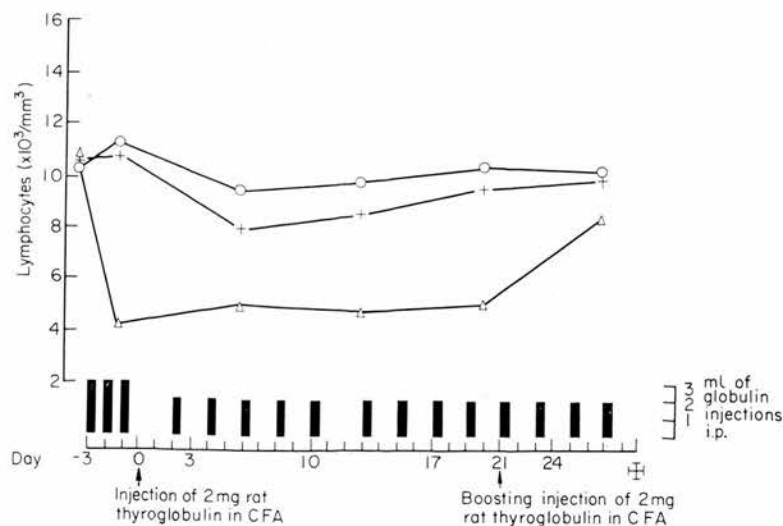


FIG. 2. The effect of various treatments on the peripheral lymphocyte count in immunized rats. Note the rapid initial fall in the animal group receiving anti-lymphocytic IgG and its eventual recovery.  $\Delta$ , Horse anti-lymphocyte globulin; +, normal horse IgG;  $\circ$ , no treatment.

Although the ALS-IgG suppressed the formation of anti-thyroglobulin antibodies, it failed to suppress the development of circulating antibodies against itself. The antibody titres against horse immunoglobulin determined by the tanned red cell method were equally high in rats treated with ALS-IgG or NH-IgG (Table 3).

TABLE 3. Tanned cell titres of antibodies to horse immunoglobulin in rats after 4 weeks treatment with NH-IgG or ALS-IgG

No. of rats	Treatment	No. of rats and titre of antibodies			
		<1:20	1:20	1:1280	1:2560
10	NH-IgG			10	
10	ALS-IgG			9	1
19	None	18	1		

The spleen and lymph nodes of one of the ALS-IgG treated rats showed lymphocytic depletion and a decrease in the number of germinal centres. Histological examination of the kidneys showed no abnormalities in any of the experimental animals.



## DISCUSSION

Chronic thyroiditis in man is closely associated with autoimmune phenomena in which the mechanisms of humoral antibody formation and of delayed hypersensitivity are involved (Glynn & Holborow, 1965). In animals, thyroiditis can be produced experimentally by sensitization with thyroglobulin in complete Freund's adjuvant (Witebsky & Rose, 1956; Terplan *et al.*, 1960; Jones & Roitt, 1961).

Anti-lymphocytic serum is known to suppress the production of delayed hypersensitivity and of primary antibody formation (reviewed by James, 1967, 1968). The observations reported in the present paper confirm that this is so in relation to autoimmune experimental thyroiditis in rats. These observations are in keeping with studies previously reported on autoimmune allergic encephalomyelitis in guinea-pigs (Waksman *et al.*, 1961; Leibowitz *et al.*, 1968). ALS has also been shown to prevent the development of Coombs positivity in NZB mice if treatment is commenced at 2 months of age (Denman *et al.*, 1967). In contrast, however, these investigators also showed that ALS was ineffective in suppressing Coombs positivity in NZB mice once this has developed. Furthermore, anti-lymphocytic-globulin treatment also failed to prevent the onset of renal disease in (NZB  $\times$  NZW) F<sub>1</sub> hybrid mice (Denman, Denman & Holborow, 1966). Indeed, in animals treated with ALS, the albuminuria was more pronounced than in control animals receiving normal IgG. However, as suggested by Denman *et al.* (1966) it is possible that ALS treatment commencing at an earlier age than 3 months could prove to be effective. ALS has also been shown to prevent the development of Freund's adjuvant arthritis in rats (Currey & Ziff, 1966, 1968) but again was relatively ineffective in suppression of already established disease.

Studies are now in progress in our laboratory to determine whether ALS has any significant effect on previously induced experimental thyroiditis in rats. It may be anticipated from the known properties of ALS that it might have relatively little effect on circulating thyroglobulin antibody titres. In this context, it is of particular interest that Leibowitz *et al.* (1968) noted that the clinical signs of autoimmune allergic encephalomyelitis could be suppressed by the administration of ALS, but there was no change in the appearance of the brain lesions including perivascular infiltration of inflammatory cells. The same authors were also able to demonstrate that treatment of already paralysed animals with ALS was capable of prolonging the survival time in four out of eleven animals with marked improvement of the physical manifestations. Again there was no improvement of the brain lesions.

The rapid initial fall in the peripheral lymphocyte count of animals following ALS administration and subsequent recovery, even during prolonged treatment, has been widely observed (reviewed by James, 1968). Recently these observations have been attributed to repopulation of the lymphocyte pool by short-lived small lymphocytes, the number of long-lived immunologically competent lymphocytes remaining markedly depleted (Denman, Denman & Embling, 1968).

The rat treated by ALS-IgG or NH-IgG developed antibodies against horse IgG but these antibodies had no effect on the immunosuppressive properties of ALS-IgG. In spite of the high antibody titres against horse immunoglobulin, there were no signs of serum sickness or of anaphylactic reactions during the course of treatment. No histological changes were observed under light microscopy in the kidneys of the ALS-IgG treated rats.

In man, ALS treatment is associated with a number of local and systemic complications, the most troublesome being severe pain at the site of the injection and thrombocytopenia

(Starzl *et al.*, 1967; Kashiwagi *et al.*, 1968) and in dogs there is evidence of nephrotoxicity (Iwasaki *et al.*, 1967). Therefore, the use of ALS in patients with autoimmune diseases of the organ-specific type (such as chronic thyroiditis) should await the further study of its application in established experimental thyroiditis in animals and the development of less toxic and more specific ALS preparations.

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The effect of anti-lymphocytic IgG on  
established autoallergic Thyroiditis in rats

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## THE EFFECT OF ANTI-LYMPHOCYTIC IgG ON ESTABLISHED AUTOALLERGIC THYROIDITIS IN RATS

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### SUMMARY

The autoallergic thyroiditis produced in rats by the injection of rat thyroglobulin in Freund's complete adjuvant can be suppressed by the administration of the IgG immunoglobulin fraction of heterologous (horse) anti-lymphocytic serum when treatment is started immediately after the antigen injection. In contrast anti-lymphocytic IgG failed to prevent the development of thyroiditis when given only 4 hr after the antigen nor did it show any suppressive effect on previously induced thyroiditis. Similar results were obtained when anti-lymphocytic IgG treatment was tested for its effect on the primary immune response to rat thyroglobulin.

### INTRODUCTION

Recent investigations have clearly demonstrated that heterologous anti-lymphocytic antibody preparations will suppress the development of a number of experimentally induced or spontaneously occurring autoimmune diseases providing that the treatment is commenced prior to the induction or onset of the disease (Denman, 1969). These include autoallergic encephalomyelitis (Waksman, Arbouys & Arnason, 1961; Leibowitz, Lessof & Kennedy, 1968a, b), autoallergic thyroiditis (Kalden *et al.*, 1968), Freund's adjuvant arthritis (Currey & Ziff, 1968) and the autoimmune haemolytic anaemia of NZB mice (Denman, Denman & Holborow, 1967). In contrast to the above, anti-lymphocytic antibody has been found to be relatively ineffective in established autoimmune diseases (Denman *et al.*, 1967) or if treatment was delayed until after the administration of the inducing antigenic preparation (Currey & Ziff, 1968). However, it has recently been shown that anti-lymphocytic antibody may have certain beneficial effects in established autoimmune diseases for it was found to protect guinea-pigs from the paralysis accompanying experimentally induced allergic encephalomyelitis even though it did not significantly suppress the histological lesions (Leibowitz *et al.*, 1968a, b). Because of these observations and the

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growing use of anti-lymphocytic antibody therapy in the treatment of autoimmune disorders in humans (Pichlmayr *et al.*, 1968; Traeger *et al.*, 1968; Trepel *et al.*, 1968) it seemed desirable to study the effect of this material on another established autoimmune disease namely, autoallergic thyroiditis in rats. In addition experiments have also been performed to determine the capacity of anti-lymphocytic antibody to prevent the development of autoimmune thyroiditis if administered shortly after the inducing antigen.

## MATERIALS AND METHODS

### *Animals*

Two to 3-month-old female rats derived from an inbred Wistar strain from Animal Suppliers (London Ltd) were used for immunization.

### *Preparation of antigen and antibody*

The preparation and properties of the rat thyroglobulin, horse anti-rat lymphocytic IgG and normal horse IgG were the same as previously described (Kalden *et al.*, 1968). The reciprocal lympho-agglutination and lymphocytotoxic titres of the anti-rat lymphocyte IgG were both 256 while the normal horse IgG possessed negligible amounts of anti-lymphocyte activity.

### *Immunization schedule*

Fifty rats were injected with 0.1 ml emulsion containing 2 mg rat thyroglobulin preparation in Freund's complete adjuvant (CFA) into a hind footpad. Immediately after the antigen injection each animal received  $2 \times 10^9$  *Bordetella pertussis* organisms intradermally into the dorsum of the same foot. Ten control animals were immunized with 0.1 ml saline in CFA and were also injected with  $2 \times 10^9$  *B. pertussis* organisms.

### *Treatment schedule*

The rats were divided into six groups and treated as shown in Table 1. Animals in Groups 1 and 2 were injected intraperitoneally with 2 ml of 1 g/100 ml anti-lymphocytic (A) or normal horse (B) immunoglobulin G. The initial injection was given at the same time (Groups 1A and 1B) or 4 hr after (Groups 2A and 2B) the injection of the rat thyroglobulin and thereafter on alternate days, the rats receiving a total of eight injections. Animals of Groups 3 and 4 received daily intraperitoneal injections for 7 days of 2 ml of 1 g/100 ml normal horse IgG or of 1 g/100 ml anti-lymphocytic IgG solution respectively commencing on day 17 after immunization while rats of Group 5 received a similar course of treatment with normal sterile saline. The control rats (Group 6) which were immunized with saline in CFA received no treatment.

### *Detection of circulating antibodies*

Tanned sheep cells were sensitized with one of the following antigens: (1) 2 mg/ml rat thyroglobulin, (2) 2 mg/ml solution of normal horse IgG, or (3) 5 mg/ml solution of anti-lymphocytic IgG, and incubated overnight at room temperature with doubling dilutions of this sera from the test and from the control animals (Herbert, 1967).

The indirect Coons test was also employed to detect antibody to thyroglobulin. Goat

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anti-rat  $\gamma$ -globulin (Hyland Division, Travenol Laboratories Inc., Los Angeles) was conjugated with fluorescein-isothiocyanate and incubated with cryostat sections of rat thyroid 4–6  $\mu$  thick and fixed in methanol at 56°C for 3 min. The conjugation of the anti-serum with fluorescein isothiocyanate was done as previously described (Kalden *et al.*, 1969b). The protein-fluorescein ratio was 0.5. The immunofluorescence method was also used with unfixed sections of rat thyroid to detect thyrocytoplasmic antibody in the test sera.

## Histology

The thyroid gland, thymus, spleen and kidneys were fixed in formol saline (4%) and sections were stained by haematoxylin and eosin (H & E).

The scoring of thyroiditis was as follows:

- = normal thyroid structure;
- = focal thyroiditis;
- ++ = more severe thyroiditis;
- +++ = diffuse thyroiditis with altered thyroid structure; and
- ++++ = severe thyroiditis with goitre.

The arithmetic mean score for each group of animals was obtained by totalling the number of '+'s for the thyroid lesions and dividing by the number of animals. The thyroid sections were randomized before they were read.

Absolute lymphocyte counts were obtained from total white cell haemocytometer counts and differential counts on Pappenheim stained blood smears and are expressed as counts per cubic millimeter blood.

## RESULTS

From Table 1 and Fig. 1(a) it will be observed that treatment with anti-lymphocytic IgG

TABLE 1. The effect of anti-rat-lymphocytic serum (ALS-IgG) on the development and on previously induced experimental autoallergic thyroiditis in rats

Group	Sensitization* (using CFA)	No. of animals	Treatment	Start of treatment	Days between sensitiza- tion and killing	Mean Score of thyroid lesions†	Significance between the different groups‡
1A	Rat thyroglobulin	5	ALS-IgG	$\pm 0$	17	0.2	$P < 0.01$
1B		5	NH-IgG			2.0	NS§
2A	Rat thyroglobulin	5	ALS-IgG	+4 hr	17	2.1	NS
2B		5	NH-IgG			2.0	NS
3	Rat thyroglobulin	10	ALS-IgG	+17 days	24	2.4	NS
4	Rat thyroglobulin	10	NH-IgG	+17 days	24	2.3	NS
5	Rat thyroglobulin	10	Saline	+17 days	24	2.4	NS
6	Saline	10	—	—	24	0.3	$P < 0.001$

\* Plus  $2 \times 10^9$  *B. pertussis* organisms.

† Gradation of thyroid lesions as described under 'Materials and methods'.

‡ Student's *t*-test.

§ NS, Not significant.

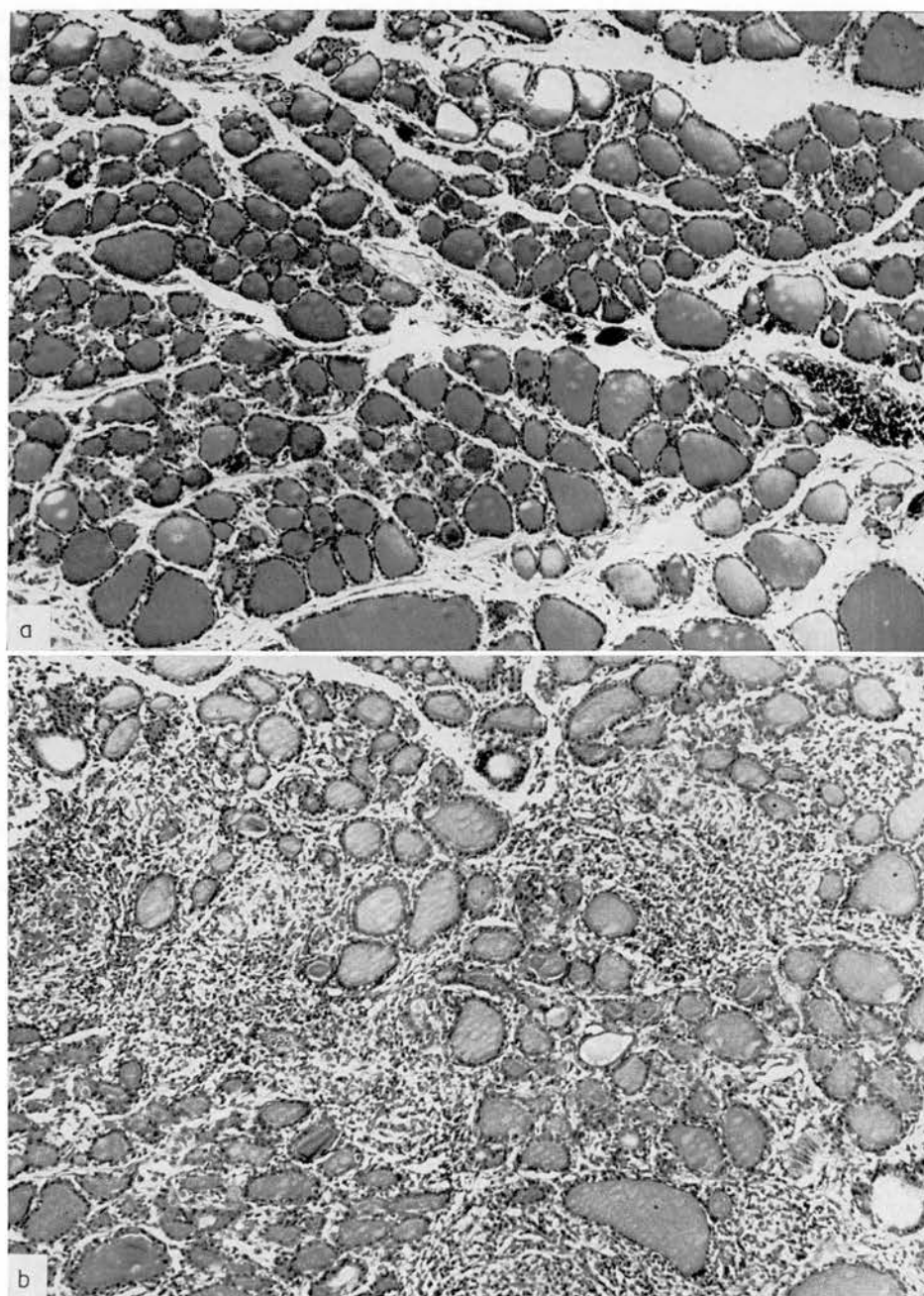


FIG. 1. (a) Thyroid of a rat after a course of treatment with horse anti-rat lymphocytic IgG started immediately after immunization with 2 mg rat thyroglobulin in CFA. The thyroid shows an intact structure. (b) Thyroid of a rat after a course of treatment with horse anti-rat lymphocytic IgG started 4 hr after immunization with 2 mg rat thyroglobulin in CFA. There is a marked inflammatory reaction with involvement and destruction of the follicles. H & E,  $\times 92$ .



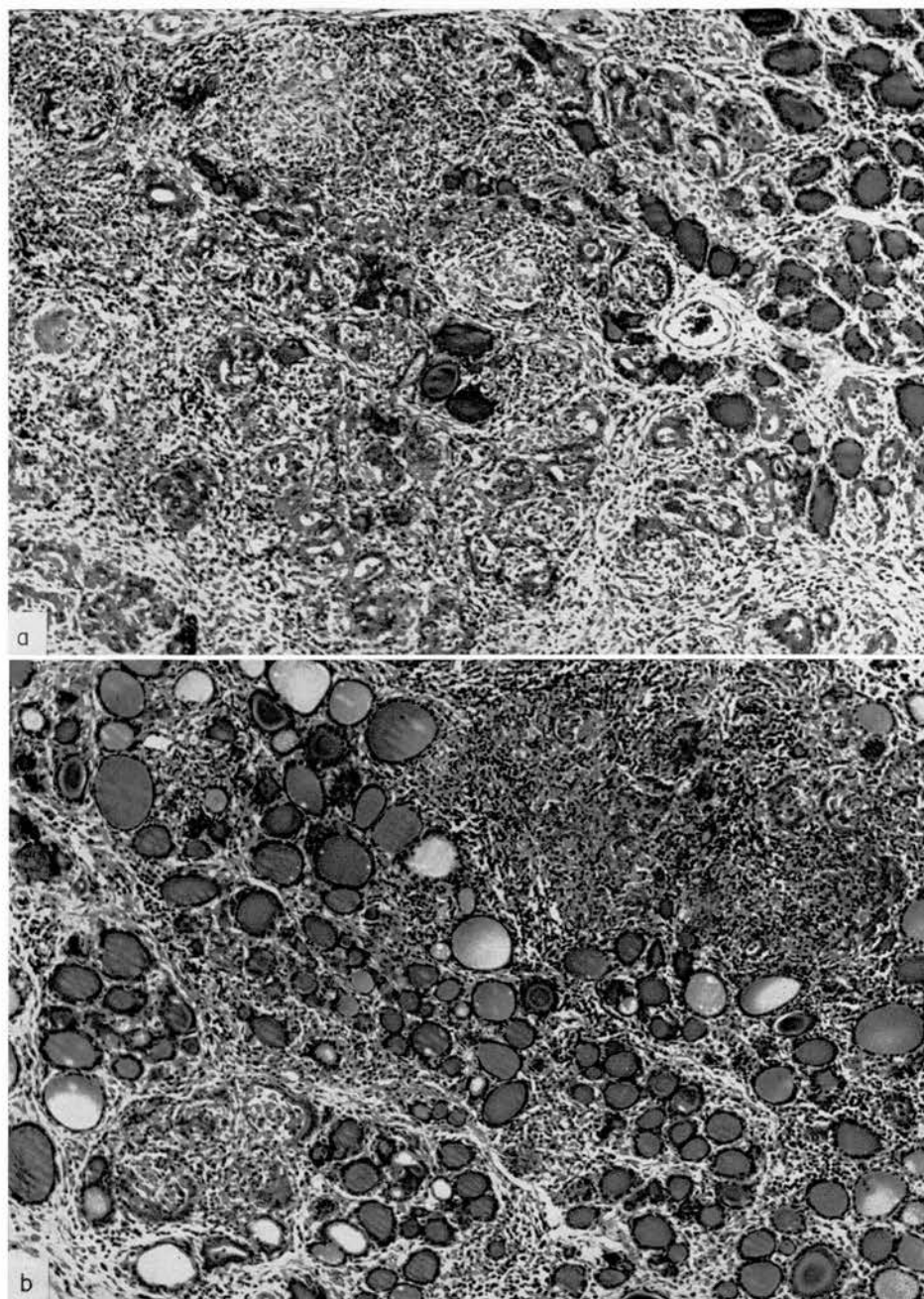


FIG. 2. (a) Thyroid of a rat after a course of treatment with sterile saline commencing 17 days after immunization with 2 mg of rat thyroglobulin in CFA. (b) Thyroid of a rat after a course of treatment with horse anti-rat lymphocyte IgG commencing 17 days after immunization with 2 mg of rat thyroglobulin in CFA. Note in both pictures the similar degree of severe inflammatory reactions with involvement and destruction of follicles. H & E,  $\times 92$ .

prevented the development of thyroiditis in rats in response to sensitization with thyroglobulin in CFA provided this was started immediately after antigen administration (mean score of thyroid lesions 0.2). In contrast anti-lymphocytic IgG failed to suppress the development of autoallergic thyroiditis when treatment was delayed until 4 hr after immunization (mean score of thyroid lesions 2.1) (Fig. 1b). Furthermore anti-lymphocytic IgG did not show any suppressive effect on already established thyroiditis when it was injected daily for 1 week starting on day 17 after the immunization, the incidence and severity of the thyroid lesions being similar to that observed in normal IgG and saline treated controls (mean score 2.3–2.4 in all groups) (Table 1 and Fig. 2a and b).

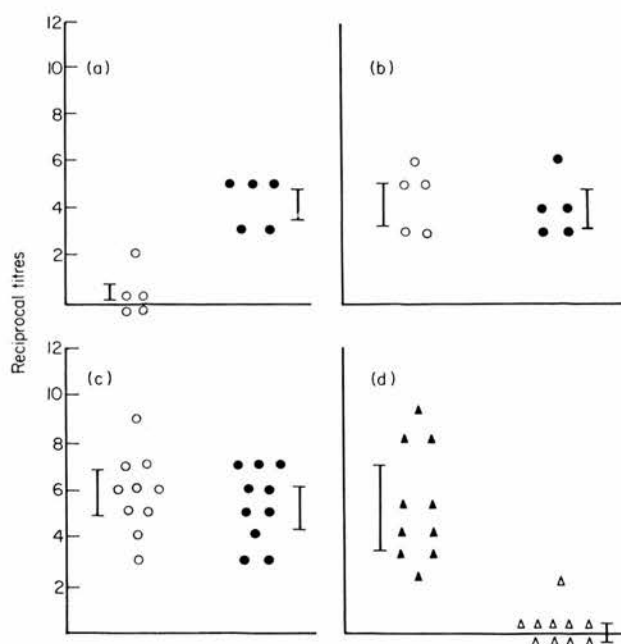


FIG. 3.  $\log_2$  titres of circulating antibodies to rat thyroglobulin in rats immunized with rat thyroglobulin in CFA and treated with horse immunoglobulins G as follows: (a) treatment started immediately after the antigen injection, (b) 4 hr and (c) 17 days after the immunization. Note that a significant ( $P < 0.01$ ) suppression of humoral antibody formation to rat thyroglobulin was achieved only in those rats in which treatment was commenced immediately after the antigen injection. (d)  $\log_2$  titres of circulating antibodies in rats immunized with rat thyroglobulin in CFA and treated with saline and in untreated control animals immunized with saline in CFA.  $\circ$ , ALS-IgG;  $\bullet$ , NH-IgG;  $\blacktriangle$ , saline;  $\triangle$ , untreated control.

Comparable results to the effect of anti-lymphocytic IgG treatment on the early developing phase of thyroiditis or on established thyroiditis were obtained when its effect was tested on the circulating antibody response to rat thyroglobulin. Thus a significant suppression of the humoral response was only achieved in those rats in which anti-lymphocytic antibody treatment was initiated immediately following thyroglobulin administration. On the other hand in those animals where treatment was not commenced until 4 hr or 17 days after immunization the tanned cell titres were not significantly different ( $P > 0.5$ ) from those observed in rats treated with normal horse IgG (Fig. 3).

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TABLE 2. Tanned red cell titres of antibodies to horse immunoglobulin in rats after treatment with ALS-IgG or NH-IgG

Group(s)	No. of rats	Treatment	Period of treatment (days)	*No. of injections	No. of rats and titre of antibodies			
					<1:20	≥1:20	1:80	≥1:160
1A and 2A	10	ALS-IgG	17	8		1	3	6
1B and 2B	10	NH-IgG	17	8		5	2	3
3	10	ALS-IgG	7	7	3	7		
4	10	NH-IgG	7	7	2	6	2	
5 and 6	20	None	—	—	20	—		

N.B. The antibody titres were determined in serum samples obtained on the day of killing (see Table 1).

\* Two millilitres of 1 g/100 ml solution of horse anti-rat-lymphocytic IgG (ALS-IgG) or normal horse IgG (NH-IgG).

The findings with the indirect immunofluorescence test using fixed rat thyroid sections confirmed the results of the tanned red cell method. Staining of the thyroid colloid was noted with the sera of the animals immunized with thyroglobulin in CFA except in those rats in which anti-lymphocytic IgG treatment had been started simultaneously with the antigen injection. Sera of the control animals gave negative results. Immunofluorescence using unfixed sections did not detect antibody against rat thyroid cytoplasm in any of the test or control animals.

Using the sensitized tanned cell technique circulating antibodies to equine IgG were detected on the day of killing in the sera of all groups of rats treated with normal or anti-lymphocytic IgG of equine origin (see Table 2).

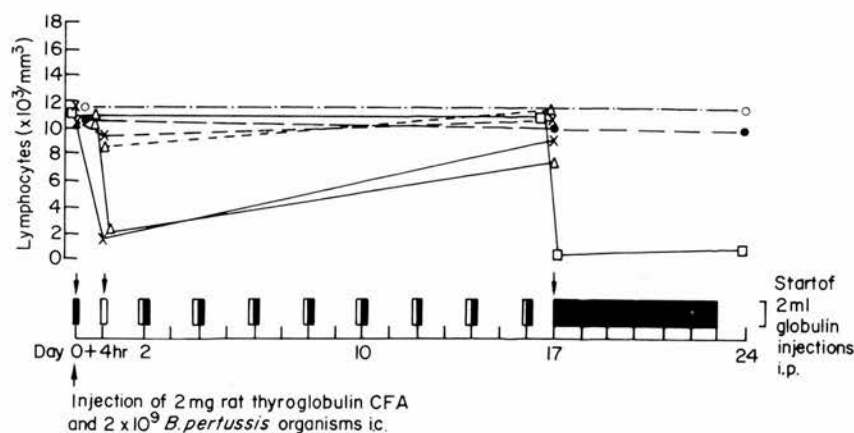


FIG. 4. The effect of ALS-IgG (—), NH-IgG (---) and saline (— · — · —) treatment on the peripheral lymphocyte count in immunized rats. Note the rapid initial fall in the animal groups receiving anti-lymphocytic IgG and its eventual recovery. The various courses of treatment were commenced; immediately following ( $\times$ , Groups 1a and 1b), 4 hr after ( $\Delta$ , Groups 2a and 2b), or 17 days after antigenic challenge ( $\square$ ,  $\bullet$ ,  $\circ$ , Groups 3, 4 and 5, respectively).

The effect of the various courses of anti-lymphocytic IgG treatment on the peripheral blood lymphocyte count is illustrated in Fig. 4.

Four hours after a single injection of anti-lymphocytic IgG, there was a significant fall in the peripheral lymphocyte count but this depression rose to almost normal levels within 17 days in spite of continuous treatment with anti-lymphocytic IgG.

Finally, light microscopy of spleen, thymus and kidney specimens did not reveal any histological abnormalities in any of the experimental animals.

## DISCUSSION

In animals, thyroiditis can be produced experimentally by sensitization with thyroglobulin in Freund's complete adjuvant (Terplan *et al.*, 1960; Jones & Roitt, 1961). In experimental thyroiditis, delayed hypersensitivity is likely to play a major part in the manifestation of the thyroid lesions (Roitt & Doniach, 1967). The development of experimental thyroiditis in rats can be enhanced by the injection of pertussis vaccine immediately after the antigen injection (Kalden *et al.*, 1969a). The fact that the rats injected with *B. pertussis* did not develop a blood lymphocytosis is explained by the use of the intracutaneous route of the vaccine injection (Morse, 1965).

Anti-lymphocytic serum (ALS) is known to suppress the production of delayed hypersensitivity and of primary antibody formation (reviewed by James, 1967a, 1969). This has been confirmed in relation to the development of experimental encephalomyelitis (Waksman *et al.*, 1961; Leibowitz *et al.*, 1968a, b), of experimental thyroiditis (Kalden *et al.*, 1968) and of Freund's adjuvant arthritis (Currey & Ziff, 1968) when ALS treatment was started before or at the time of immunization with the appropriate antigen in CFA. In contrast, however, ALS was ineffective in suppressing Coombs positivity in NZB mice once this has developed (Denman *et al.*, 1967) and furthermore ALS also failed to prevent the onset of renal disease in (NZB  $\times$  NZW) $F_1$  hybrid mice when treatment was commenced at the age of 3 months (Denman, Denman & Holborow, 1966).

The experimental results presented in this paper clearly demonstrate that the daily injection of anti-lymphocytic antibody for the period of 1 week had no significant effect on previously induced experimental thyroiditis. ALS also failed to suppress the development of thyroiditis when treatment was started 4 hr after the immunization with thyroglobulin in CFA. Only when the injection of anti-lymphocytic antibody was commenced immediately after the antigen injection could the development of thyroid lesions be prevented. These findings are in keeping with results reported by Currey & Ziff (1968) that delayed ALS treatment had only little effect on the manifestation of Freund's adjuvant arthritis. Although Leibowitz *et al.* (1968a, b) found that in experimental allergic encephalomyelitis ALS treatment given during a period of 10–18 days after the immunization protected the animals from paralysis, anti-lymphocytic antibody did not suppress the manifestation of histological brain lesions. Furthermore these authors showed that ALS treatment in already paralysed animals was capable of prolonging the survival time in four out of eleven animals with marked improvement of the physical manifestations. Again these animals showed histologically extensive areas of demyelination. This finding suggests that anti-lymphocytic antibody may have some suppressive effect on an established autoimmune process though the mechanism of this effect is not yet clearly understood.

The failure of anti-lymphocytic antibody treatment to suppress the formation of humoral



antibody to thyroglobulin when injected 4 hr or 17 days after antigen administration confirms the results of previous investigators in other systems (Berenbaum, 1967; James 1967b).

On the basis of the present results it appears once more that anti-lymphocytic antibody has limited access to central lymphoid tissue (Denman *et al.*, 1967; Levey and Medawar, 1967; Denman & Frenkel, 1968) being relatively incompetent in inactivating memory cells or preventing plasma cell proliferation in these sites (see review Denman, 1969). Nevertheless, it is possible that more prolonged courses of anti-lymphocytic antibody treatment combined with other forms of immunosuppression, including cytostatic drugs and other antisera (Willoughby & Coote, 1966) may be of value in treating established autoimmune diseases in man.

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CHAPTER X

CHAPTER XSTUDIES ON IMMUNOLOGICAL TECHNIQUES

"A Latex Particle Precipitation Test in the Diagnosis of Thyroid Disease"

(1962) J. clin. Path., 15, 148-152

J.R. PHILP  
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X : 1-6

"Automated Determinations of Thyroid and Gastric Complement Fixing Antibody: Comparison with the Fluorescent Antibody and Manual Complement Fixation Methods"

(1966) Automation in Analytical Chemistry 1966. Technicon International Symposium p. 347-353.

W.J. IRVINE  
X : 7-14

"Automated Determinations of Thyroid and Gastric Complement Fixing Antibody; Comparison with the Fluorescent Antibody and Manual Complement Fixation Methods"

(1966) Clin. exper. Immunol., 1, 341-354

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X : 15-29

"The application of microphotometry to the indirect immunofluorescent antibody technique using the human gastric parietal cell antigen antibody system"

(1969) Clin. exper. Immunol., 5, 607-617.

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# **A LATEX PARTICLE PRECIPITATION TEST IN THE DIAGNOSIS OF THYROID DISEASE**

BY

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## A latex particle precipitation test in the diagnosis of thyroid disease

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**SYNOPSIS** The method of latex particle precipitation has been applied to the detection of circulating antibody to a crude extract of thyroid. The sensitivity of the method is intermediate between that of the agar diffusion and the tanned cell haemagglutination techniques. If only the grosser degrees of latex precipitation are taken as being significant, the method would appear to be of equal value as the agar diffusion test in the diagnosis of lymphadenoid goitre.

The use of inert particles such as latex, bentonite, and collodion has provided an additional simple method for detecting circulating antibody. The particles are coated with the appropriate antigen and the presence of the corresponding antibody is indicated by agglutination or precipitation of the particles.

Polystyrene latex particles have been used in this way by Singer and Plotz (1956, 1958) and Olsen and Rantz (1958) for the detection of rheumatoid factor and more recently by Christian, Mendez-Bryan, and Larson (1958) and Fessel (1959) for the detection of LE factor, and by Kelen and Labzoffsky (1960) for the detection of antibodies to *Leptospira*.

The purpose of this paper is to evaluate the usefulness of antigen-coated latex particles in the detection of thyroid antibodies and to compare this method with the established procedures of tanned cell haemagglutination and agar gel diffusion.

### LABORATORY METHODS

**SALINE EXTRACT OF THYROID AND LIVER** Human thyroid tissue was obtained at thyroidectomy or from a fresh cadaver. Liver tissue was likewise obtained at an early necropsy. The tissues were frozen immediately in a vacuum flask surrounded by an alcohol and solid carbon dioxide mixture. They were then cut into small pieces and homogenised in the cold for two minutes at high speed in an M.S.E. homogeniser with an equal volume of saline. After the addition of a further volume of saline the homogenate was frozen and thawed three times and left to extract overnight at 4°C. The coarse sediment was removed by centrifugation for five minutes at 2,000 r.p.m.

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in an M.S.E. major refrigerated centrifuge, and the supernatant centrifuged at 59,310 *g* in a preparative Spinco. The supernatant was used as the antigen. It was stored at -20°C.

**POLYSTYRENE LATEX** A suspension of Difco latex particles, 0.81  $\mu$  in diameter, was used as supplied by Baird & Tatlock, Ltd., London.

**GLYCINE BUFFER** Glycine in 1% NaCl 0.1 M, was adjusted to pH 8.2 with a solution containing 2.5 ml. N/1 NaOH/litre and with 10% citric acid. This pH was found to give optimal precipitation of the antigen-coated latex particles.

**LATEX TEST** After preliminary studies using dilutions of antigen (thyroid extract) up to 1:1,000 it was found necessary to use dilutions of 1 in 5, 1 in 10, and 1 in 25. Maximal precipitation for a given serum was found to take place with any one of these three dilutions in an unpredictable manner. Dilutions of antigen higher than 1 in 25 in no case enhanced the degree of precipitation.

The antibody in the form of test serum was used in an undiluted state, since dilution of the serum did not increase the degree of precipitation. The sera had been stored at -20°C. for two to three years and had been inactivated for previous diagnostic tests. Further heating was not carried out.

Dilutions of the latex suspension of 1 in 10 and 1 in 20 were made in the glycine buffer. The 1 in 10 latex dilution was used to prepare the stock suspension of antigen-coated latex particles by mixing with an equal volume of each of the three antigen dilutions. The mixtures were then shaken and left on the bench for 30 minutes to allow adsorption of the antigen to take place. With the addition of merthiolate to 1 in 10,000 these mixtures were stable at 4°C. for at least one month. Two drops of the antigen-latex suspension at each dilution of the antigen were

then transferred to 3 in.  $\times$   $\frac{1}{2}$  in. tubes and two drops of undiluted serum were added to each. After shaking, the mixture was drawn up into capillary tubes which were placed in plasticine racks. The 1 in 20 dilution of uncoated latex suspension was used to set up a control test for each test serum.

The capillary tubes were kept on the bench and examined after three, 24, and 48 hours for precipitation. No further precipitation occurred after 48 hours. Incubating at fixed temperatures in the range of 4° to 56°C. was not found to enhance the degree of precipitation. The degrees of precipitation were designated by the digits 0, 1, 2, 3, and 4, according to the length of the fragmented column of precipitated particles, 0 signifying no precipitation and 4 maximal precipitation, with 1, 2, and 3 intermediate degrees respectively (Fig. 1). The degree of precipitation was apparent in the majority of cases overnight but was finally read at 48 hours. The strongest degree of precipitation observed in any of the three tubes was taken as the final reading.

**GEL DIFFUSION TEST** The agar double diffusion technique (Oudin, 1948) was used as modified by Oakley and Fulthorpe (1953).

**TANNED RED CELL TEST** The test using thyroglobulin-coated sheep red cells was that used by Roitt and Doniach (1958) after Rose and Witebsky (1956). Purified thyroglobulin prepared after the method of Derrien, Michel, and Roche (1948) was used.

#### CLINICAL MATERIAL

The sera were selected from specimens which had been kept after examination for thyroid antibodies for diagnostic purposes (Irvine, 1960a). No attempt is made in the present study to determine the incidence of positive immunological tests in unselected thyroid patients. Each serum was given a number and all tests were carried out in ignorance of the diagnosis and the results of other tests. The following sera were used:—

Fifty-three sera from 32 histologically proven cases of lymphadenoid goitre, 21 of which were obtained from 10 patients who, at the time, had had no surgical interference or only biopsy, and the remaining 32 sera were obtained from 22 patients who had had a partial or hemithyroidectomy, anything from a few days to 10 years previously.

Fifty-eight sera were from 51 cases of lymphadenoid goitre diagnosed clinically without histological proof.

Twenty-two sera were obtained from 17 cases of spontaneous adult hypothyroidism.

Thirty-six sera were from 31 patients with simple goitre, 20 of whom had subsequent histological proof.

Twenty sera were from 16 cases of carcinoma of the thyroid, all histologically proven but sera obtained either before or after treatment.

Twenty-nine sera were from 22 cases of thyrotoxicosis.

Two sera were from two cretinous children.

Four sera were from four patients with subacute (de Quervain's) thyroiditis. In two cases histology was available.

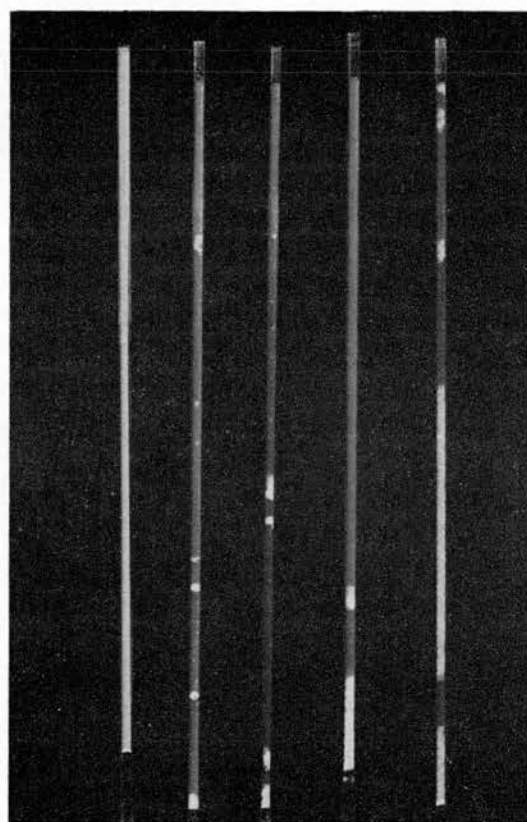


FIG. 1. The five capillary tubes illustrated are selected from tests described in the text using various negative and positive sera at the different antigen dilutions. Reading from left to right the tubes have been arranged to show degrees of precipitation graded as 0, 1, 2, 3, and 4. The degree of precipitation is assessed according to the length of the fractionated column of precipitated particles. To the naked eye the consistency of the latex is that of a suspension in tube 0 and is clearly floccular in tube 4, although this is difficult to illustrate in a photograph.

Fifty-one sera were from 51 cases of rheumatoid arthritis. All these sera had strongly positive Rose-Waaler tests.

Six sera were from six cases of systemic lupus erythematosus (S.L.E.).

Seventy sera were from 70 healthy antenatal patients (normal controls).

#### RESULTS

Table I shows the distribution of 0, 1, 2, 3, and 4 reactions in the latex capillary test. It will be seen that while none of the 70 normal sera precipitated the coated particles some degree of precipitation

TABLE I  
DISTRIBUTION OF REACTIONS IN LATEX CAPILLARY TEST

Diagnosis	No. of Sera	Degrees of Latex Agglutination				
		0	1	2	3	4
Lymphadenoid goitre (clinically $\pm$ histology)	111	32	17	10	7	45
Spontaneous hypothyroidism	22	7	5	7	—	3
Simple goitre	36	27	5	4	—	—
Carcinoma thyroid	20	15	3	2	—	—
Thyrotoxicosis	29	20	4	1	2	2
Cretins	2	2	—	—	—	—
Subacute thyroiditis	4	2	1	—	—	1
Rheumatoid arthritis	51	39	5	5	2	—
S.L.E.	6	2	—	4	—	—
Normal (ante-natal)	70	70	—	—	—	—
Totals	351	216	40	33	11	51

TABLE II

Tanned Cell Haemagglutination Titre	SERA GIVING POSITIVE AGAR DIFFUSION TESTS														
	Lymphadenoid Goitres (Clinically $\pm$ Histology)			Spontaneous Hypothyroidism			Simple Goitre			Carcinoma Thyroid			Thyrotoxicosis		
	No. of Sera	No. with 3 to 4 Latex Reactions	No. with Positive Agar Diffusion Test	No. of Sera	No. with 3 to 4 Latex Reactions	No. with Positive Agar Diffusion Test	No. of Sera	No. with 3 to 4 Latex Reactions	No. with Positive Agar Diffusion Test	No. of Sera	No. with 3 to 4 Latex Reactions	No. with Positive Agar Diffusion Test	No. of Sera	No. with 3 to 4 Latex Reactions	No. with Positive Agar Diffusion Test
0	12	—	—	4	—	—	8	—	—	7	—	—	4	—	—
10	9	—	—	2	—	—	11	—	—	3	—	—	4	—	—
30	8	—	—	4	—	—	6	—	—	1	—	—	2	—	—
90	17	2	1	1	—	—	6	—	—	3	—	—	3	—	—
270	11	2	2	1	—	—	3	—	—	1	—	—	7	—	—
810	8	4	3	2	—	—	2	—	—	—	—	—	4	—	—
2,430	6	6	5	2	1	1	—	—	—	1	—	—	1	1	1
7,290	11	11	9	1	—	—	—	—	—	1	—	—	1	—	1
21,870	8	7	7	3	—	—	—	—	—	2	—	—	—	—	—
65,610	21	20	21	2	2	2	—	—	—	1	—	—	3	3	3
Totals	111	52	48	22	3	3	36	—	—	20	—	—	29	4	5

was caused by a proportion of the sera from each of the other groups studied. It is also apparent that the highest proportion of sera giving three and four reactions occur in the lymphadenoid goitre group while lesser degrees of precipitation (0-2) are encountered in other types of thyroid diseases.

Table II shows the number of sera in each group of patients that gave a positive agar diffusion test for thyroglobulin antibody. They correspond closely with the number of sera giving three and four latex reactions. Five sera gave a 3 to 4 latex reaction, but a negative agar diffusion test, while two sera gave a positive agar diffusion test but did not give 3 or 4

latex precipitation. This table also compares the latex and agar diffusion tests with the tanned cell haemagglutination test using thyroglobulin-coated sheep red cells. It will be seen that the positive agar diffusion test and the 3 + 4 latex reactions are distributed towards the higher end of the tanned cell haemagglutination titre scale. The tanned cell haemagglutination titres are widely distributed in thyroid disease, and it is of interest that three cases of carcinoma of the thyroid gave a high titre and yet both the agar diffusion and the latex capillary tests were negative. It is also of interest that two sera from one patient with histological proof and two sera from



TABLE III

Tanned Cell Haemagglutination Titre	LYMPHADENOID GOITRE HISTOLOGICALLY PROVEN					
	Biopsy Only or before Thyroidectomy			Up to 10 years Post-thyroidectomy		
	Patients	3 to 4 Latex Reactions	Positive Agar Diffusion Test	Patients	3 to 4 Latex Reactions	Positive Agar Diffusion Test
0	1	—	—	6	—	—
10	1	—	—	3	—	—
30	—	—	—	—	—	—
90	1	—	—	4	—	—
270	1	—	—	2	—	—
810	1	1	1	1	—	—
2,430	1	1	1	1	1	—
7,290	1	1	1	1	1	1
21,870	3	3	3	4	3	4
Totals	10	6	6	22	5	5

two cases diagnosed clinically as having lymphadenoid goitre gave tanned cell haemagglutination titres of 270 or less, but still gave positive 3 and 4 latex reactions.

A small proportion of sera from patients with rheumatoid arthritis and S.L.E. precipitated thyroglobulin-coated latex particles (1, 2, and 3 reactions), but such sera can be distinguished by the fact that they will also cause precipitation of the uncoated latex control particles or of latex particles coated with human liver antigen. Such non-specific precipitation did not occur with any of the 232 thyroid sera in spite of the fact that the incidence of rheumatoid arthritis and of lupus erythematosus antinuclear factor is known to be raised in lymphadenoid goitre (Buchanan, Crooks, Alexander, Koutras, Wayne, and Gray, 1961; Weir, Holborow, and Johnson, 1961). These results, together with the absence of any degree of precipitation by the 70 normal sera, indicate the specificity of the test for thyroid antibodies.

The main clinical interest attached to the detection of thyroid antibodies is in the diagnosis of lymphadenoid goitre. The results in this disease are shown in Table III, where the patients are divided into two series. First, a small series of 10 patients from whom sera were obtained before thyroidectomy and, secondly, a larger series of 22 patients in whom serological tests were carried out at various intervals after surgery. It is again seen that there is a close parallel between the agar diffusion test and the latex method.

#### DISCUSSION AND CONCLUSIONS

A method of using latex particles for the detection of thyroglobulin antibody is described which is both extremely cheap and easy to perform. The time of reading the test is shorter than with the agar diffusion method, in which eight days should be allowed to elapse before pronouncing the result to be negative. The sensitivity of the capillary latex

method for the detection of thyroglobulin antibodies is appreciably greater than that of the agar diffusion test, but markedly less than the tanned cell haemagglutination test. However, when only three and four reactions are taken into account as being significant the sensitivity of the method and its specificity within the range of thyroid diseases make the capillary latex test and the agar diffusion test of equal diagnostic value in lymphadenoid goitre.

No figures can as yet be given to indicate the true incidence of positive capillary latex tests in the various thyroid disorders. In the case of lymphadenoid goitre this would require the serological test to be done before treatment in patients in whom the diagnosis was subsequently established on the basis of adequate histology. As far as we are aware no such series of patients has yet been published even for the agar diffusion test. In the present series the incidence of positive agar diffusion tests of 60% in those lymphadenoid goitre patients who had not had thyroidectomy and of some 25% in those patients in whom thyroidectomy had been performed up to 10 years previously is consistent with current views. A similar incidence is found with the capillary latex test when 3 to 4 reactions are used.

The main objections to the capillary latex technique is the interpretation of what constitutes a grade 1 or 2 precipitation as opposed to grades 3 or 4. With experience of the method this difficulty is readily overcome and consistent results obtained. An improved method of grading might be to measure the total length of the discrete fragments in a positive column (see Fig. 1). Failure to grade the test results in loss of diagnostic specificity for lymphadenoid goitre. The same problem arises with the tanned red cell technique where there is uncertainty in selecting an arbitrary titre of diagnostic value in lymphadenoid goitre. On the other hand the agar diffusion test in the majority of cases gives a clear positive or negative result although a weak reaction can cause considerable difficulty.

It is necessary to carry out control tests using uncoated latex particles to exclude the possibility of obtaining false positive results with rheumatoid or systemic lupus erythematosus sera.

In conclusion, while the tanned cell haemagglutination technique (Roitt and Doniach, 1958) and the complement-fixation test (Trotter, Belyavin, and Waddams, 1957) remain as the techniques for the sensitive and quantitative measurement of circulating thyroid antibodies, the capillary latex method may prove to be of value as a rapid screening test in cases suspected clinically of having lymphadenoid goitre, possessing as it does the combined virtues of cheapness, ease of performance and readability, and a specificity for this disease equal to that of the agar diffusion test. Diagnostically, however, the capillary latex method is likely to have the same limitations as the agar diffusion test because it will fail to reveal those patients with Hashimoto disease with negative or insignificant titres of thyroglobulin antibody but high titres of circulating antibody directed against intracellular antigen detected by the method of complement fixation (Trotter *et al.*, 1957) or cytotoxicity (Irvine, 1960b, 1961a, 1961b). Conversely the complement-fixation test *per se* would not detect those patients who only possess antibody to thyroglobulin. It would therefore appear that a combination of these two types of test is required for diagnostic purposes.

Our thanks are due to Professor Robert Cruickshank for his interest in the development of the capillary latex test.

We are also indebted to Professor Sir Derrick Dunlop and Dr. J. S. Robson for allowing us to study their patients, and to the many other clinicians (in particular, Dr. W. R. M. Alexander of the Rheumatic Unit at the Northern General Hospital) for kindly sending us sera.

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(66-II, P-82)

**Automated Determinations of Thyroid and  
Gastric Complement Fixing Antibody; Comparison with the  
Fluorescent Antibody and Manual Complement Fixation Methods**

W. J. Irvine

# Automation in analytical chemistry 1966



TECHNICON INTERNATIONAL · Ardsley (Chauncey), New York 10502

# Automated Determinations of Thyroid and Gastric Complement Fixing Antibody

## Comparison with the Fluorescent Antibody and Manual Complement Fixation Methods

W. J. IRVINE

Gastric and thyroid serological tests have become part of the routine investigations of haematological and thyroid problems in clinical medicine. Although there are a number of gastric and thyroid antibodies, those that are detectable by the method of complement fixation are among the most significant in the diagnosis of atrophic gastritis and chronic thyroiditis (1, 5).

The basis of the complement fixation test is that certain types of antigen-antibody reaction are characterised by the fixation of complement. The fixation of complement is detected *in vitro* by an indicator system consisting of sheep red cells and haemolysin. The haemolysin will only destroy the red cells if complement is still freely available. The

Takatsy microtitre apparatus is recommended for doing manual complement fixation tests (2). Although this method is in many ways satisfactory, the results in different laboratories and in the same laboratory may vary on account of the subjective interpretation of the end point and because of the mental fatigue that occurs when large numbers of sera are to be tested. Two papers have been published on the automation of the complement fixation test for the detection of gastric and thyroid antibodies (2, 3). The present paper reports the results of further studies.

Figure 1 shows the currently recommended flow diagram. Antigen, complement and antibody, each in separate channels, are fed into the Analyzer

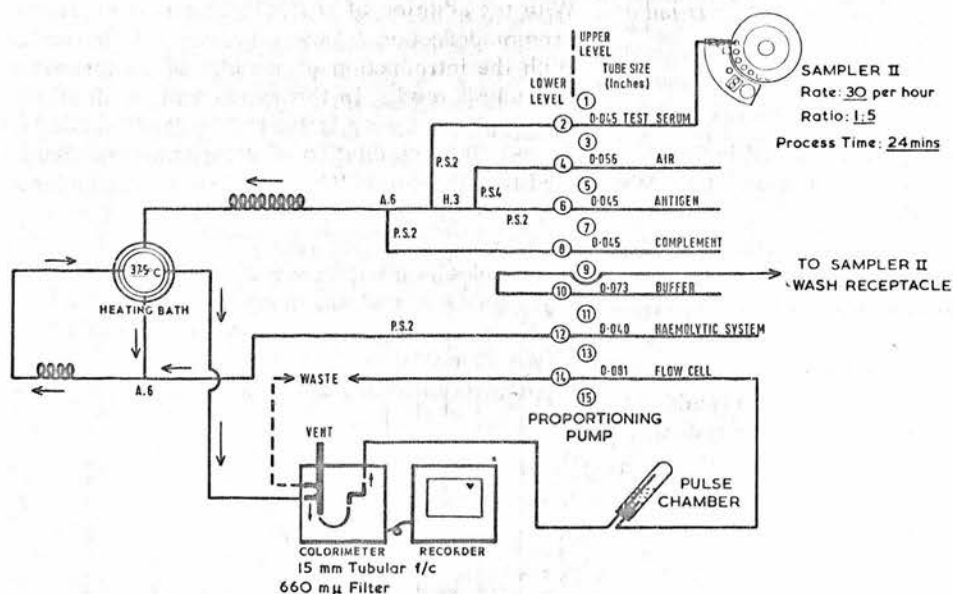


Figure 1: Flow Diagram

Presented at the European Technicon Symposium, »Automation in Analytical Chemistry«, Paris, Nov. 2, 3, 4, 1966, by W. J. Irvine, Medical Research Council, Clinical Endocrinology Research Unit; Endocrine Clinic and Department of Therapeutics, Royal Infirmary; Edinburgh, Scotland, Great Britain.

through a 10 roller Proportioning Pump. These reagents are then brought together and mixed in a double mixing coil for 2¼ minutes. They pass to a 37.5°C thermostat (oil bath) with a delay coil to encourage the antigen-antibody reaction to take place and for complement to be fixed. The flow rate is such that the incubation time is 9 minutes. After this stage sheep red cells sensitised with haemolysin



(haemolytic system) are introduced. Following a further period of mixing (single mixing coil) and incubation at 37.5°C the degree of lysis of the red cells is detected by a photocell using a turbidity filter with a transmission of 660 mμ. Figure 2 shows the fitting of the flow cell debubbler that has been developed. This is helpful in obtaining a maximal flow to the flow cell with minimum risk of carrying over small bubbles of air which would marr the recorder tracing.

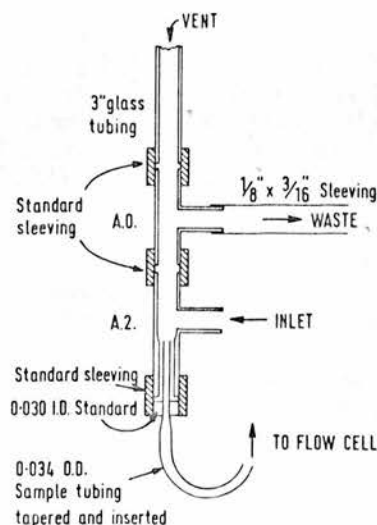


Figure 2: Detail of Debubbler

### Reagents

**Complement** (guinea pig). Preserved in Richardson's solution (Stayne's Laboratories, High Wycombe, Bucks., England).

**Diluent.** Veronal buffer at pH 7.2 (Oxoid BR 16). This diluent is filtered prior to use.

**Sheep red blood cells** in Alsever solution (Stayne's Laboratories).

**Rabbit anti-sheep haemolytic serum** (Stayne's Laboratories). 0.08% haemolytic serum is added to 1% erythrocytes to form the haemolytic system.

**Antigen.** Saline extracts of various tissues as previously described (2).

Known positive sera for the antibodies being studied.

**Tween 20.** 1 drop per 500 ml diluent.

30 sera are tested per hour with a sample to wash ratio of 1:5. The whole process from start to finish takes 24 minutes. It is essential to have a perfect bubble pattern and to wash out the machine after use with a detergent solution (*e.g.* Decon 75) followed by a prolonged wash with distilled water overnight. From time to time the plastic tubing should be replaced and the glassware (including the flow

cell) washed with dichromate cleaning fluid and thoroughly rinsed.

### Calibration of Photocell

Figure 3 shows the calibration of the apparatus for varying concentrations of red cells passing through the photocell (from 2% down to 0.05% red cells and 2% cells that have been fully lysed). The recorder system is sensitive to small reductions in the concentration of red cells in suspension but is insensitive to the amount of haemoglobin released as a result of lysis of the red cells.

### Complement Fixation Test

The complement fixation is done in 3 stages:

- (a) Titration of complement
- (b) Titration of antigen
- (c) Testing of sera for complement fixing antibody.

### Titration of Complement

To do a complement titration serial dilutions of complement may be placed in the automatic sampler. Diluent is substituted for antigen and for antibody. As shown in Figure 4 the recorder is adjusted so that it reads at the lower end of the scale when only buffer is going through the machine. With the addition of red cells the recorder gives a prompt deflection to the other end of the scale. With the introduction of samples of complement, haemolysis results. In this experiment the dilutions of complement were 1: 10, 15, 20, 25, 30, 32.5, 35, 40 and 50. Each dilution of complement was sampled for 20 seconds. The complement titration was

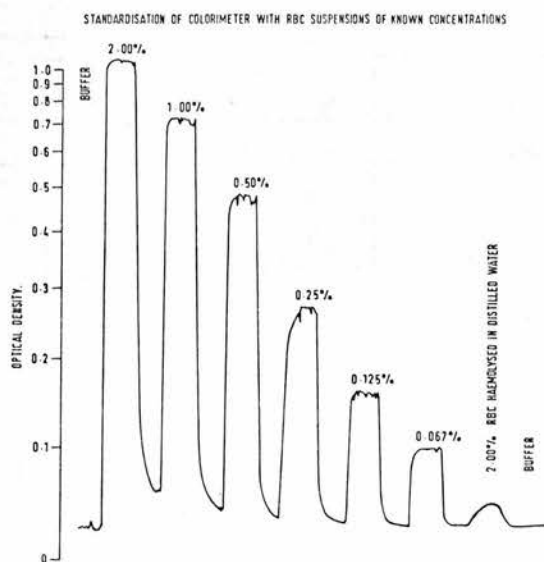


Figure 3: Standardisation of Colorimeter

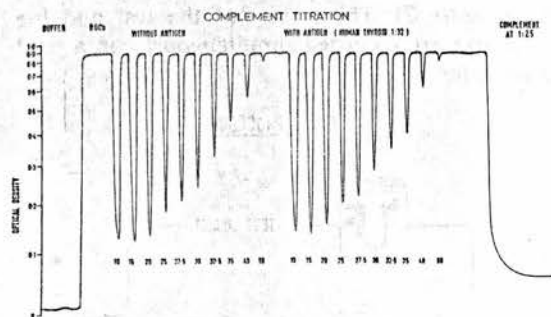


Figure 4: Complement titration by intermittent sampling

then repeated in the presence of antigen and an almost identical tracing obtained.

One important characteristic of the AutoAnalyzer is that the haemolytic reaction for a given dilution of complement may not be maximal when it is sampled for only a short period. Thus, when a dilution of complement is sampled for 20 seconds (as for example a dilution of 1: 25), only a proportion of the red cells are lysed. If the complement at the same dilution (1: 25) is fed into the machine continuously then 100% lysis is obtained over a period of 6 minutes. This is important in selecting the appropriate complement dilution that is to be used for the titration of antigen or for the detection of antibody, for under these conditions complement is supplied to the machine continuously.

Since the reproducibility of the complement fixation test largely depends on the accuracy with which complement is titrated, it is preferable to titrate complement by continuous rather than by intermittent sampling (Figure 5). Each strength of complement is sampled continuously for 3 minutes without any wash periods. In this way the minimum haemolytic dose of complement can be determined. In this particular experiment one minimum haemolytic dose of complement was given by a complement dilution of 1: 100 using 0.25% suspension of red

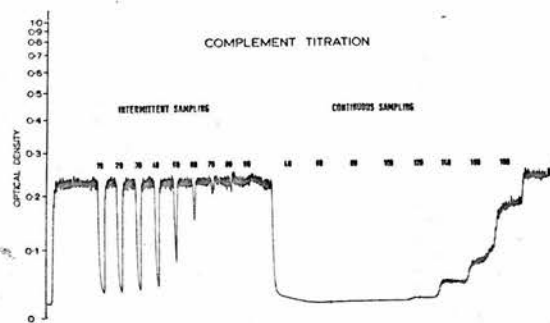


Figure 5: Complement titration: continuous sampling compared to intermittent sampling

cells sensitised with 0.02% haemolytic serum. The method of continuous sampling is much more sensitive and precise than is that of intermittent sampling. Before doing a complement titration it is important to prime the Analyzer by passing through a 1: 10 dilution of complement for 5 to 10 minutes. Complement requires to be titrated each day. During the day's testing a reservoir of complement at the appropriate dilution must be kept at 4°C in an ice bath.

#### Titration of Antigen

It is necessary to carry out a titration of the tissue extract in order to find the highest dilution of the extract that still contains sufficient antigen to fix all the available complement in the presence of excess antibody. To do this, the antigen line is attached to the automatic sampler, the cups of which contain the tissue extract in doubling dilutions. The complement line is placed in a reservoir of the appropriate complement dilution (chosen to provide 1.5 minimum haemolytic dose) and the antibody line is placed in a reservoir of a serum that is known to have a high titre of antibody for that particular antigen (manual complement fixation titre  $\geq 128$ ). Some few minutes are allowed for the system to come into equilibrium with regard to complement and antibody before introducing the samples of antigen.

Figure 6 shows the titration of a saline extract of pig gastric mucosa. It is advisable to start with the most dilute samples of tissue extract (1: 128, 64, 32, 16, 8, 4, 2) because high concentrations of protein-

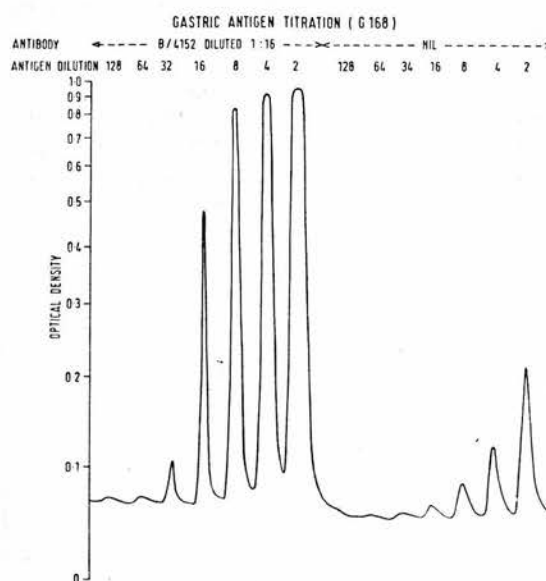


Figure 6: Titration of gastric antigen

aceous material may well result in precipitation within the machine with resultant interference with the turbidity measurements of the colorimeter. The known positive serum used as a source of gastric complement fixing antibody had a titre in the manual of  $\geq 512$ ; this serum was diluted 1: 16 before use. The antigen titration should be repeated in the absence of antibody to check on the nonspecific absorption of complement and the turbidity of the different antigen dilutions. When sampled for 20 seconds an antigen dilution that causes considerable but not total fixation of complement in the presence of excess antibody is suitable for the screening of test sera. In Figure 6 a dilution of gastric extract of 1: 16 was chosen as suitable. Gastric parietal cell antibody is not species-specific and pig gastric extract has been found suitable as antigen (4). The tissue extracts need only be titrated once, provided the extracts are stored thereafter at  $-20^{\circ}\text{C}$  in small aliquots.

#### Screening of Sera for Complement Fixing Antibody

Having titrated the complement and the tissue extracts we are now in a position to test large numbers of sera for the presence of complement fixing antibodies. Complement and antigen at appropriate dilutions are fed continuously into the Analyzer and the antibody line is attached to the automatic Sampler. The cups of the Sampler contain a series of test sera in a dilution of 1: 4.

In any complement fixation test it is essential to confirm the tissue specificity of the reaction and to exclude anti-complementary effects and errors due to the turbidity of the sera. When looking for thyroid complement fixing antibodies, for example, the sera must be tested against thyroid antigen and also in the absence of antigen or against liver extract as a control. A direct comparison between the test run and the control run can be obtained by splitting the serum sample (using fitment PT. 2) so that the test serum goes to each of two circuits arranged in pa-

rallel (Figure 7). The results of the test and the control run are recorded simultaneously on a dual pen recorder.

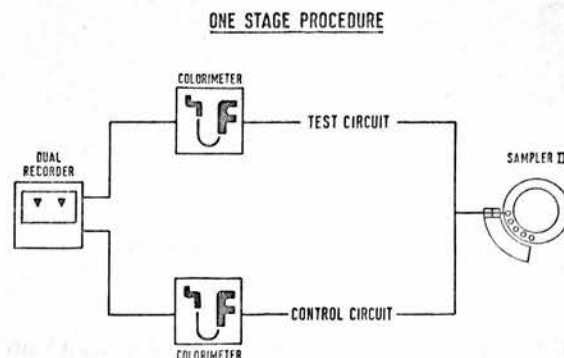


Figure 7: One stage procedure for direct comparison of test with control

In Figure 8 the upper tracing is the complement fixation test with a number of sera using thyroid extract as antigen and the lower tracing is the control run without antigen. 1.0% suspension red cells sensitised with 0.08% haemolytic serum were used in this experiment. 1.5 minimum haemolytic doses of complement were employed. There is a formalin peak at the beginning and end of the tracing. The first formalin peak is followed by a titration (1: 64, 128, 256 and 512) of a known positive serum as a standard for the thyroid antibody test. Thereafter 30 sera were tested at a dilution of 1: 4. A significant difference in the height of the peaks between the two tracings indicates that a complement fixation reaction has occurred between the test serum and the thyroid extract. When both tracings show that haemolysis has been completely inhibited, the serum should be retested in a higher dilution. Frequently, however, the shape of the peaks indicates whether complement fixing antibody in addition to anticomplementary activity may also be present.

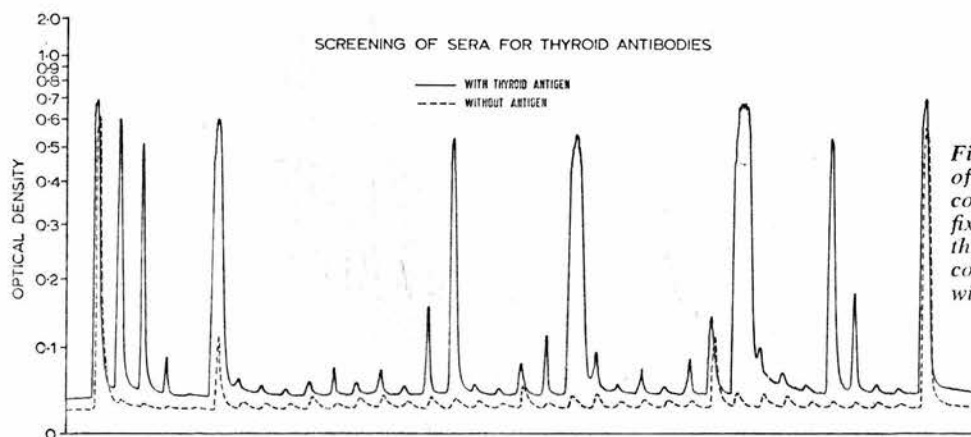


Figure 8: Recording of a series of complement fixation tests with thyroid tissue compared with controls



The sera are run through the Analyzer once more using gastric extract and liver extract to test for gastric parietal cell antibody and for tissue specificity. For screening against thyroid, stomach, liver and for anticomplementary activity and turbidity control a total of 0.2 ml serum is required.

Carry-over from a strongly positive serum to the next sample is not a common problem in the routine screening of sera from endocrine and haematological clinics with a sample to wash ratio of 1: 5. When it does occur the strongly positive serum should be titrated (see Figure 9) and the following serum retested. To avoid carry-over, the flow diagram could be altered so that the antigen-antibody-complement reaction took place in the sampler cups. Sampler cups would then require to be incubated on the Sampler II at 37°C. The antigen and complement could be dispensed into the cups from reservoirs via the Proportioning Pump using transmission tubing of suitable calibre. In addition to avoiding carry-over such a modification might lead to considerable saving of antigen and complement. The criticism of such a modification might be that (1) the smaller the volumes of reagents used the bigger the error in the dispensing of these reagents into the sampler cups and that (2) mixing may be inadequate. In the present study, however, carry-over was not a serious problem and the supply of suitable human thyrotoxic glands and of pig gastric mucosa provided an adequate source of antigen. Likewise, there was no difficulty in the supply of complement, although to use it in smaller quantities would have been more economical.

## Results

Tables I and II show the correlation between the height of the peak on the AutoAnalyzer tracing and

the titre obtained with the same serum in the manual complement fixation test in a study of gastric and thyroid complement fixing antibodies, respectively. In this study the flow diagram used was that described in an earlier publication (2) using a 2% erythrocyte suspension and complement titrated by the intermittent method. Sera that gave a good titre

Table I. Gastric Complement Fixation Test

Indirect fluorescent antibody method (undiluted serum)	Complement fixation		No. of sera
	Manual (titre)	AutoAnalyzer (sera at 1: 4 dilution) (height of peak)	
++	≥ 32	+++	27
		++	10
		+	2
		Negative	—
++	≥ 4 ≤ 16	+++	—
		++	2
		+	9
		Negative	1
+	≥ 4 ≤ 16	+++	—
		++	1
		+	8
		Negative	6
+	< 4	+++	—
		++	—
		+	3
		Negative	8
Negative	< 4	+++	—
		++	—
		+	—
		Negative	155

Difference in peak heights given by test and control sera on the AutoAnalyzer.

≥ 45 units = +++

≥ 12 < 45 units = ++

≥ 3 < 12 units = +

Using percentage transmission paper.

TITRATION OF SERA FOR CF THYROID ANTIBODY

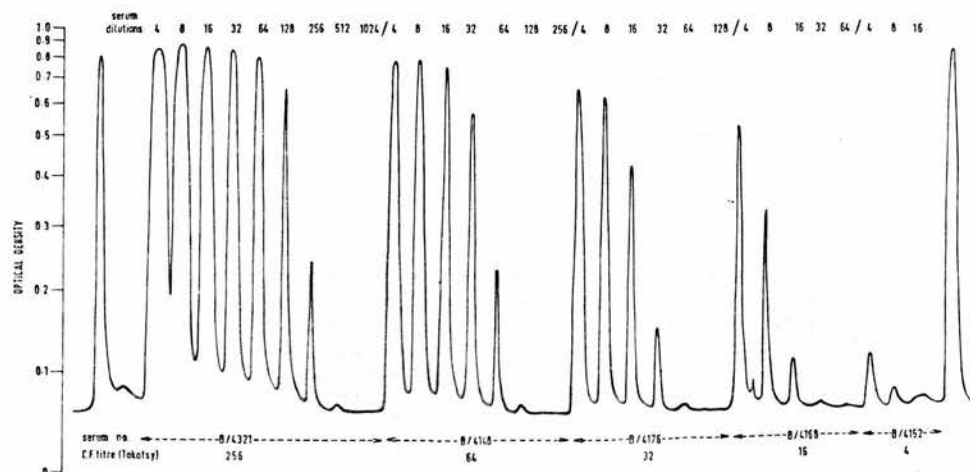


Figure 9: Titration of sera for thyroid complement fixing antibody



Table II. Thyroid Complement Fixation Test

Indirect fluorescent antibody method (undiluted serum)	Complement Fixation		No. of sera
	Manual (titre)	AutoAnalyzer (sera at 1:4 dilution) (height of peak)	
++	≥32	+++	34
		++	7
		+	3
++	≥4≤16	Negative	—
		+++	2
		++	3
		+	5
+	≥4≤16	Negative	2
		+++	—
		++	2
		+	4
+	<4	Negative	5
		+++	—
		++	—
		+	6
Negative	<4	Negative	21
		+++	—
		++	—
		+	—
		Negative	138

Difference in peak heights given by test and control sera on the AutoAnalyzer.

≥ 45 units = +++

≥ 12 < 45 units = ++

≥ 3 < 12 units = +

Using percentage transmission paper.

in the manual method (≥ 1: 32) gave a strong peak on the AutoAnalyzer using 2% red cells. The reproducibility of the manual method is ± one doubling dilution. It is therefore not surprising that there is some discrepancy in the results between the two methods with weakly positive sera. When compared to the immunofluorescent technique for detecting the same antibodies, no false positives were

recorded by the AutoAnalyzer for either thyroid or gastric complement fixing antibodies in the 309 sera tested. A similar study using the flow diagram as shown in Figure 1 with 10% red blood cells and using 1.5 minimum haemolytic doses of complement is presently in progress.

#### Titration of positive Sera

Test sera giving a strong organ-specific reaction in a dilution of 1: 4 can be titrated. Figure 9 illustrates the titration by doubling dilution of 5 sera for thyroid antibodies by the Analyzer using the flow diagram previously published (2). There is a formalin peak at the beginning and end of the tracing to assist localisation. The titres determined in this manner correspond very closely with those obtained by the manual technique (Takatsy) which are also included in Figure 9 (1: 256; 1: 64; 1: 32; 1: 16; 1: 4, respectively).

**Acknowledgements:** I am grateful to Mr. Kenneth Marwick, F.I.M.L.T., for his invaluable technical assistance. Figs. 3, 4, 6 and 8 and Tables I and II are reproduced by permission of the Editor and Publisher of Clin. exper. Immunol.

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#### Résumé

Les auteurs ont mis au point une technique automatique pour le dépistage et le titrage des anticorps sériques fixant le complément, produits en réponse à des antigènes gastriques (cellules pariétales) et thyroïdiens microsomatiques. La méthode automatique fournit une sensibilité comparable à celle de la technique manuelle de fixation du complément, mais elle est un peu moins sensible que la méthode indirecte des anticorps fluorescents. Par rapport à la technique manuelle de fixation du complément, la méthode automatique présente l'avantage d'une précision et d'une reproductibilité bien supérieures et supprime tout facteur d'interprétation personnelle. Elle réclame moins d'habileté de la part du technicien. La méthode automatique utilise seulement de faibles quantités de sérum mais elle consomme une quantité considérable d'antigène et de complément. Dans la présente étude, on a utilisé un antigène d'origine humaine.

**Summary**

An automated technique has been established for the screening and titration of sera for complement fixing antibody to gastric (parietal) cell and thyroid microsomal antigens. The automated method is of equal sensitivity to the manual complement fixation technique but slightly less sensitive compared to the indirect fluorescent antibody method. The automated method has the advantage over the manual complement fixation test of much greater accuracy and reproducibility and the element of subjective interpretation is removed. It is less demanding on technician skill. The automated method requires only small amounts of sera but it consumes considerable amounts of antigen and of complement. Human antigen has been used in the present study.

**Zusammenfassung**

Ein automatisches Verfahren für die serienmäßige Bestimmung und Titration von Seren für komplementbindende Antikörper auf gastrische Zellen und auf zur Schilddrüse gehörende Plasmosom-Antigene wurde entwickelt.

Die automatische Methode entspricht der Empfindlichkeit der manuellen Komplementbindungsreaktion, zeigt jedoch eine geringere Empfindlichkeit beim Vergleich mit der indirekten Fluoreszenz-Antikörpermethode. Die automatische Methode hat über die manuelle Komplementbindungsreaktion den Vorteil größerer Genauigkeit und Reproduzierbarkeit. Das Element der subjektiven Interpretation ist somit entfernt worden. Die Methode erfordert wenig Geschick vom technischen Personal, kleine Serenmengen, verbraucht jedoch beträchtliche Mengen an Antigen und Komplement. Zu den hier vorliegenden Studien wurde menschliches Antigen angewandt.

Automated determinations of thyroid and gastric complement-fixing antibody; comparison with the fluorescent antibody and manual complement-fixation methods

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## AUTOMATED DETERMINATIONS OF THYROID AND GASTRIC COMPLEMENT-FIXING ANTIBODY; COMPARISON WITH THE FLUORESCENT ANTIBODY AND MANUAL COMPLEMENT-FIXATION METHODS

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### SUMMARY

An automated technique has been established for the screening and titration of sera for complement-fixing antibody to gastric (parietal) cell and thyroid microsomal antigens. The automated method is of equal sensitivity to the manual complement-fixation technique but slightly less sensitive compared to the indirect fluorescent antibody method. The automated method has the advantage over the manual complement-fixation test of greater accuracy and reproducibility and the element of subjective interpretation is removed. It is less demanding on technician skill. The automated method requires only small amounts of sera but it consumes a considerable amount of antigen. Human antigen has been used in the present study.

The automated method for complement-fixation reactions has some advantages over the manual method for both routine clinical purposes and for the research laboratory.

A positive test for complement-fixing autoantibody to thyroid (Trotter, Belyavin & Waddans, 1957) has been shown to correlate in man with histological evidence of chronic thyroiditis (Roitt & Doniach, 1960; Buchanan *et al.*, 1962; Senhauser, 1964). Likewise, the presence of gastric complement-fixing antibody in the serum (Irvine *et al.*, 1962; Taylor *et al.*, 1962; Irvine, 1963) correlates with histological and functional evidence of chronic gastritis (Irvine *et al.*, 1965; Irvine, 1965). The detection of these autoantibodies is of value to the clinician when thyroid disease or atrophic gastritis is suspected. They are also of value in studying the familial aspects and the natural history of thyroid and gastric disorders. As both thyroid and gastric disorders are common, the number of sera which the clinical laboratory may be asked to examine may be large. An automated technique would therefore be valuable. The second reason for attempting to automate the complement-fixation method is to obtain better standardization in the technique itself. A preliminary report has been made (Irvine, 1966).

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## METHODS

*Automated Complement-Fixation Method*

Technicon Auto-Analyzer equipment was used (Technicon, Chertsey, Surrey, England; Chauncey, New York) and is illustrated in Fig. 1. It consists of an automatic sampler, proportioning pump (10 roller), double coil oil bath, colorimeter and recorder. The principle of the method is the same as the manual complement-fixation technique (*vide infra*) but the reagents are drawn into a system of transmission tubing where they are mixed and incubated. The rate of transmission through the system is determined by the internal diameter of the transmission tubing that is placed in the manifold of the proportioning pump. Mixing is achieved by putting the samples through a coil of glass tubing with the longitudinal axis

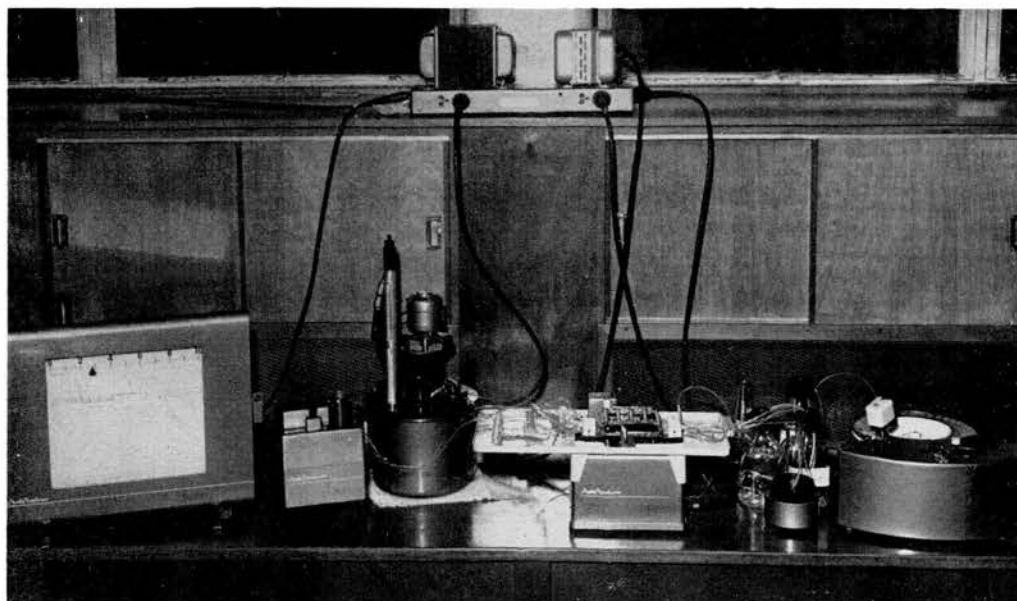


FIG. 1. The Auto-Analyzer consisting of (from right to left) the automatic sampler, proportioning pump, double coil oil incubating bath, colorimeter and recorder.

horizontal. Incubation without additional mixing is achieved using a glass coil with a vertical longitudinal axis. The duration of mixing and of incubation is determined by the length and the diameter of the tubing used. The internal diameter of the tygon plastic transmission tubing and of the glass mixing and incubating coils was 1.6 mm. Polyethylene tubing of 0.034 mm internal diameter was used to convey the individual reagents to the proportioning pump.

The samples to be tested were placed in the individual cups of the automatic sampler, while the other reagents were used continuously at a constant rate. The automatic sampler can be adjusted for the number of samples tested per hour and also for the period of sampling and for the period of washing between different samples. The fluid within the trans-

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mission tubing is broken up into short segments by the introduction of bubbles of air at frequent and constant intervals; this minimizes diffusion of reagents within the system. The ratio of fluid to air in the system should be approximately 2:1, but the air must be removed before the samples pass through the colorimeter. 0.2 ml *Brij* was added to each 500 ml of complement-fixation test diluent to act as a detergent and to adjust the surface tension of the fluid passing through the Auto-Analyzer so that a correct bubble pattern was obtained.

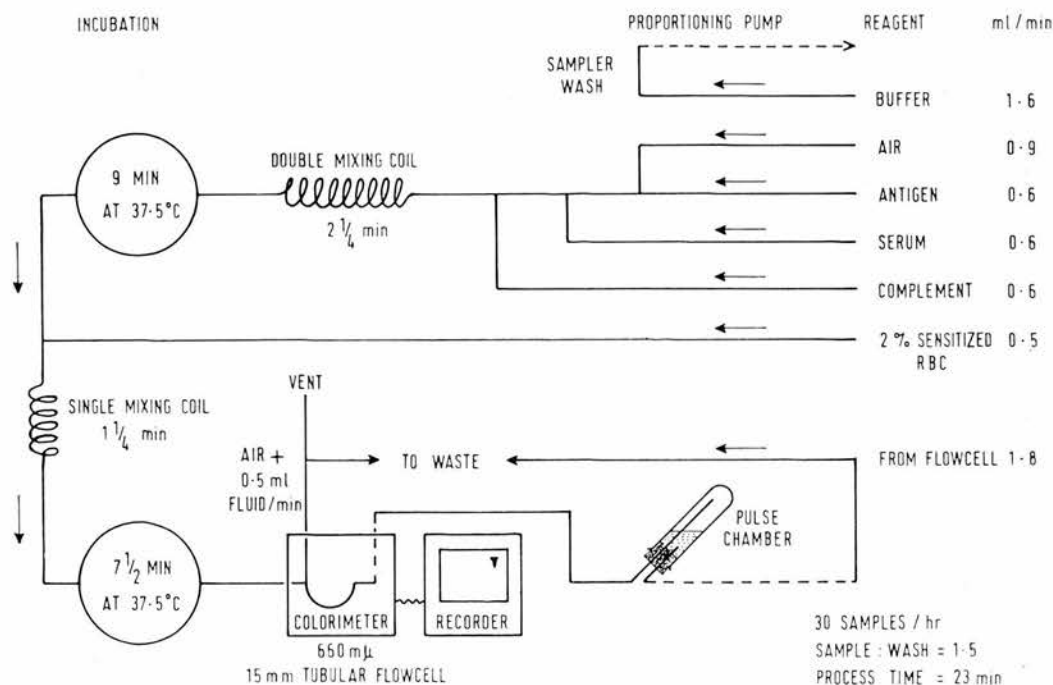


FIG. 2. The flow design for the automation of complement-fixation tests using saline tissue extracts and human sera. The approximate internal diameters of the tubes passing through the proportioning pumps in the order shown are: 0.073, 0.056, 0.045, 0.045, 0.073, 0.056, 0.040 and 0.090 in.

All reagents were added through capillary tubes using Technicon D<sub>1</sub> junction pieces. All joins in the system must be made perfectly. After each day's experiments the system was washed through with distilled water. The transmission tubing was replaced approximately every 2 weeks and all glass tubing and the flow cell was disassembled and washed with dichromate cleaning fluid and thoroughly rinsed. Attempts to wash the plastic tubing with various detergents resulted in erroneous complement titrations in spite of repeated washing with water and buffer. The flow diagram is shown in Fig. 2. The flow rates of the various reagents

was determined by direct measurement. The internal diameters (inches) of the tubing in the manifold of the proportioning pump were as follows: sampler wash, 0.073; air, 0.056; antigen, serum and complement, 0.045; re-sample, 0.073; air, 0.056; sensitized red cells, 0.040; and from flow cell, 0.090. It is to be noted that if a 5 roller proportioning pump is to be used the flow rates will be considerably greater. The success of this flow design depends on a perfect bubble pattern.

The following reagents were used in the present series of experiments: saline extracts of

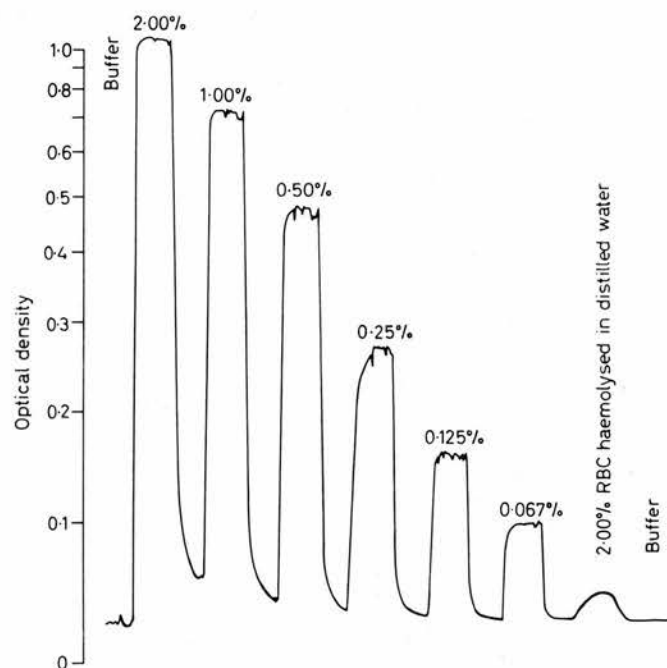


FIG. 3. Standardization of the Auto-Analyzer colorimeter with red cell suspensions of known concentrations. The colorimeter is sensitive to small changes in the concentrations of red blood cells in suspension but is insensitive to the amount of haemoglobin released as a result of lysis of the red cells.

human thyrotoxic tissue, of the mucosa of the body of human stomach obtained at partial gastrectomy for duodenal ulcer, and of rat liver; preserved guinea-pig complement (Stayne Laboratories Ltd, High Wycombe, Buckinghamshire, England); sera from patients with thyrotoxicosis, Hashimoto goitre, primary hypothyroidism, pernicious anaemia, simple atrophic gastritis and from random patients with unknown immunological status; fresh defibrinated sheep red cells obtained from the abattoir and used within 7 days; rabbit haemolytic serum (Stayne Laboratories Ltd, High Wycombe, Buckinghamshire, England); complement-fixation test diluent containing barbitone 0.575 g, sodium chloride 8.500 g, magnesium chloride 0.168 g, calcium chloride 0.028 g and barbitone soluble 0.185 g/l

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(Oxoid, London, England). The saline tissue extracts were made by obtaining the fresh tissue at operation. The tissue was cut up finely and homogenized with an equal volume of complement-fixation test diluent. Three further volumes of the diluent were then added and mixed thoroughly with the homogenate which was then filtered through a layer of eight-ply gauze and centrifuged for 10 minutes at 1150 *g*. The supernatant, which contains the antigen, was pipetted off and stored at  $-20^{\circ}\text{C}$  in 5 ml amounts. All sera were inactivated at  $56^{\circ}\text{C}$  for 30 min before use. The sheep red cells were washed in saline at least three times or until the supernatant after centrifugation was clear of haemoglobin and then centrifuged in buffer. A 2% suspension of the red cells was then sensitized with 0.16% rabbit haemolytic serum (haemolysin) at  $37^{\circ}\text{C}$  for 5 min. The cells were kept in suspension by a magnetic stirrer. The complement and the antigen in selected dilutions were kept in separate containers in an ice bath. All reagents, with the exception of the sera and diluent, were filtered through gauze before use. The diluent was filtered through Watman No. 1 paper.

As in the manual method the degree of complement fixation is assessed by the inhibition of haemolysis of the sensitized sheep red cells. In the automated method this was assessed colorimetrically using a turbidity filter (660  $\text{m}\mu$ ). The results were recorded on optical density paper. The colorimeter and recorder were calibrated using complement-fixation test diluent (buffer) alone, suspensions of sheep red cells in known concentrations and haemolysed sheep red cells (Fig. 3).

#### *Complement titration*

A complement titration was done initially in the absence of antigen; the antigen and serum pick-up lines were placed in buffer and the complement pick-up line was attached to the automatic sampler which carried a series of cups containing the appropriate dilutions of complement. The complement titration was then repeated in the presence of the antigen dilution that had been selected (*vide infra*) for the screening of test sera for the corresponding antibody. Complement titrations in the presence and absence of thyroid antigen are shown in Fig. 4.

With the flow diagram as shown in Fig. 2, the haemolytic reaction for a given dilution of complement may not be maximal due to loss in concentration of the reagent by diffusion as it passes through the system when it is sampled for only a short period. This is illustrated in Fig. 4 by the observation that when a 1:25 dilution of complement is sampled for 20 sec only a proportion of the red cells are lysed (peak at 0.18 and 0.20 optical density scale in the absence and presence of complement, respectively). But if complement at the same dilution is fed into the machine continuously then 100% lysis is obtained over a period of 6 min. This is important in selecting the appropriate complement dilution that is to be used for the titration of antigen or for the detection of antibody; under these circumstances complement is supplied to the machine continuously. The sensitivity of the method for the detection of antigen or antibody is of course dependent on the amount of complement that is made available. The dilution of complement that is appropriate for the titration of antigen or detection of antibody has been found to be that dilution which when supplied at the rate of thirty samples per hour with a sample to wash ratio of 1:5 gives a recorded deflection of 0.7 optical density divisions (e.g. in Fig. 4 from a non-lytic base line of 0.90 to a peak height at 0.20 in the presence of the antigen dilution that is to be used in subsequent tests).



A complement titre is required at the beginning of each day's experiments and once the complement dilution has been constituted it must be kept in an ice-bath to minimize complement decay.

#### *Titration of antigen*

A titration of antigen was done in order to find the highest dilution that would still contain sufficient antigen to fix all the available complement in the presence of excess antibody. The antigen line was attached to the automatic sampler, the cups of which contained antigen in doubling dilutions. The complement pick-up line was placed in a

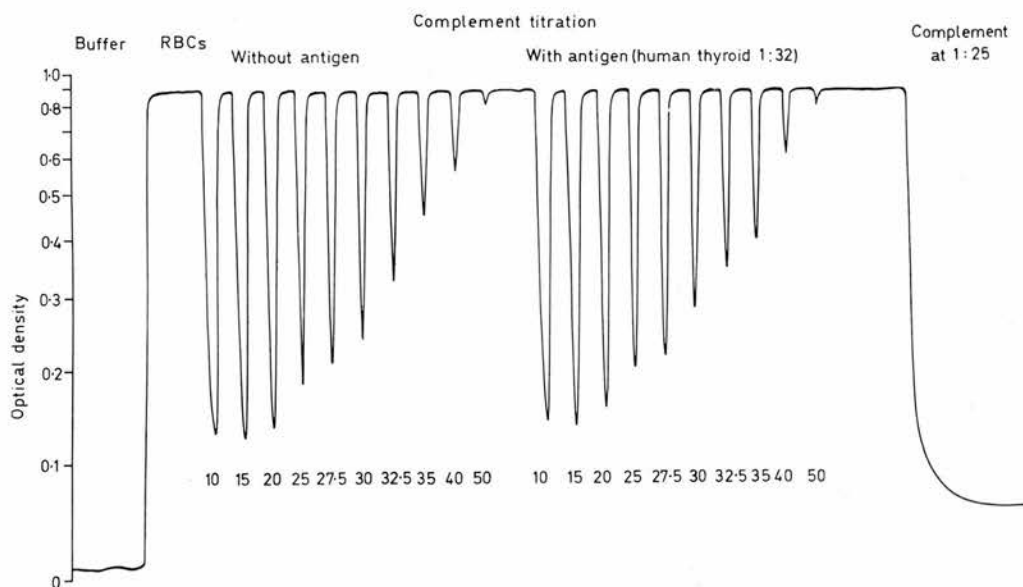


FIG. 4. Titration of preserved guinea-pig complement in the absence and presence of thyroid antigen (diluted 1:32). The dilutions of complement (1:10 to 1:50) are shown opposite each peak on the tracing. The complement titre was selected as 1:40. When the complement is sampled continuously in this dilution complete haemolysis is achieved in a period of 3 min. The difference between the colorimeter readings at the beginning and end of the tracing is due to the turbidity of the preparation of thyroid extract (see Fig. 5).

reservoir of the appropriate complement dilution and the antibody pick-up line was placed in a reservoir of a known positive serum that may be suitably diluted for the purposes of economy. Some few minutes were allowed for the system to come into equilibrium with regard to complement and to antibody before introducing the samples of antigen. This resulted in 100% haemolysis of the sensitized sheep red cells and established the base line, which was determined by the setting of the colorimeter and the turbidity of the serum. It is useful for localization purposes on the recorder paper to fill the first and last cups on the automatic sampler with 1% formalin in buffer. This results in a peak on the recorder chart at the beginning and at the end of the experiment as formalized cells are not susceptible to the lytic action of haemolysin and complement.

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Fig. 5 illustrates the titration of a saline extract of human gastric body mucosa (G168). It is advisable to start with the most dilute samples because higher concentrations of proteinaceous material may well result in precipitation with resultant interference with the turbidity measurements of the colorimeter. The antigen titration should be repeated in the absence of antibody to check on the non-specific absorption of complement and the degree of turbidity of the different antigen dilutions. When each dilution of the antigen is sampled

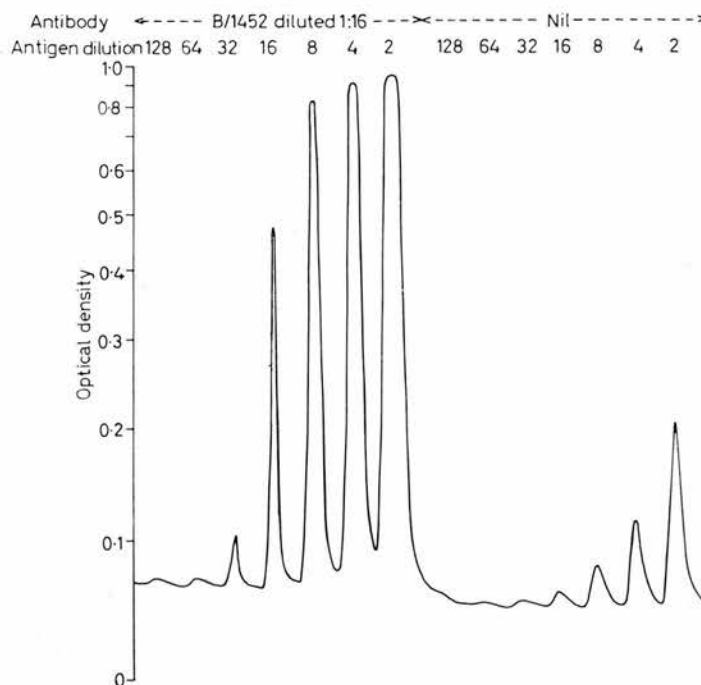


FIG. 5. Titration of a saline extract of human gastric mucosa (G.168) for antigenicity. A serum (B/4152) from a patient who was known to have high titres of gastric specific complement-fixing antibody was used in a dilution of 1:16. Complement was sampled continuously at the appropriate dilution (in this case 1:35). A control series of antigen dilutions is included without antibody to determine the non-specific binding of complement and the turbidity of the various dilutions of tissue extract. A titre of 1:16 was chosen as appropriate. When sampled continuously the antigen at this dilution would be sufficient so that the antigen-antibody reaction would be capable of fixing all the complement available.

for only 20 sec there is loss in concentration of the sample due to diffusion as it passes through the system. When sampled in this manner an antigen dilution that causes considerable but not total fixation of complement in the presence of excess antibody was found to be suitable for the screening of test sera for the corresponding antibody. In Fig. 5, for example, the dilution of antigen that was selected as suitable for the next stage in the procedure was 1:16. Gastric, thyroid and liver extracts were titrated in this manner using the corresponding organ-specific or non-organ-specific complement-fixing antibody.

Provided the saline extracts are stored in their concentrated form at  $-20^{\circ}\text{C}$  and made up to the desired dilutions when required it was not necessary to repeat the antigen titrations except at infrequent intervals.

*Screening and titration of sera for complement-fixing antibody*

With complement and antigen being continually fed into the machine from reservoirs containing these reagents at the appropriate dilutions, the antibody pick-up line was attached to the automatic sampler, the cups of which contained a series of test sera in a

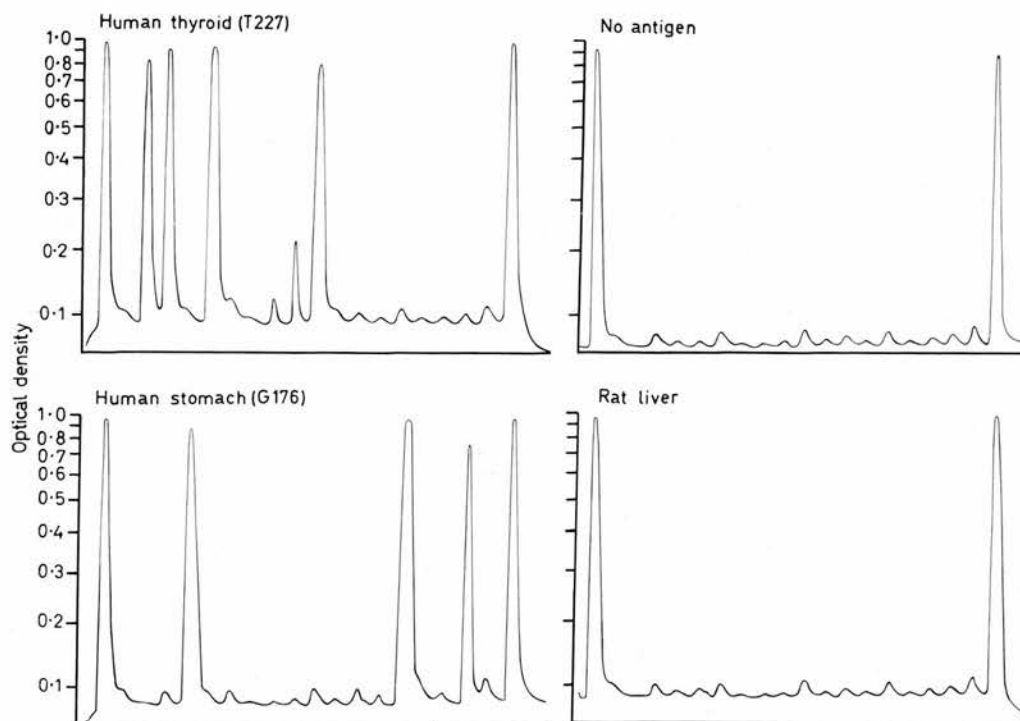


FIG. 6. The screening of eighteen sera for organ-specific complement-fixing thyroid and gastric autoantibodies using the Auto-Analyzer. The same sera were put through the machine four times with a different component on each occasion. A formalin peak is placed at the beginning and end of each run. For this initial screening test all sera were used in a dilution of 1:4 after inactivation in a water bath at  $56^{\circ}\text{C}$  for 30 min.

dilution of 1:4. As before, the first and last cup of the automatic sampler should contain 1% formalin in buffer to help localization on the recorder chart. The same series of sera were retested using different antigens and in the absence of antigen to check for organ specificity and for anti-complementary effects. An example of such a series is shown in Fig. 6. For screening against thyroid, stomach, liver and for anti-complementary activity a total of 0.2 ml serum is required.

As in the titration of complement and of antigen using the automatic sampler, the reaction

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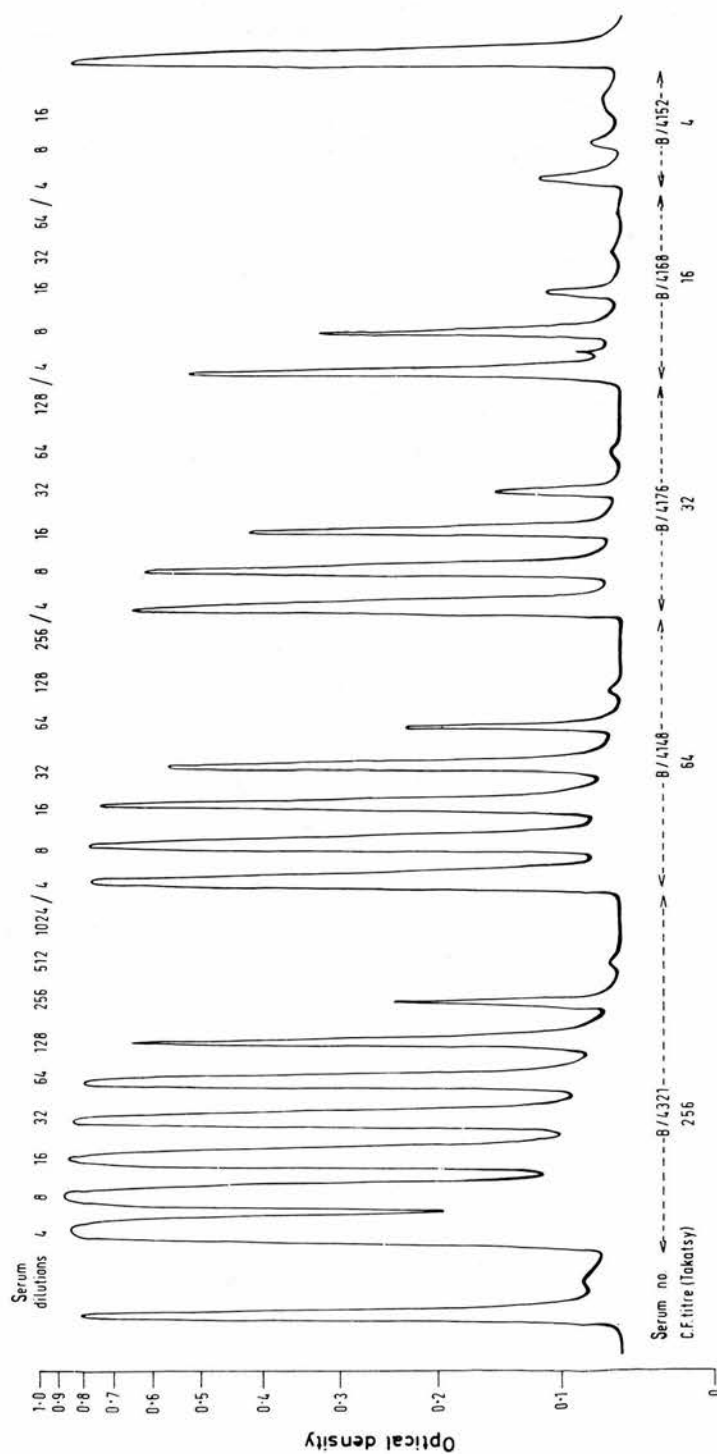


FIG. 7. The titration of sera for thyroid complement-fixing antibody. In this series human thyroid extract (T. 229) was used in a dilution of 1:32 and preserved guinea-pig complement in a dilution of 1:47.5. The test sera had previously been screened in a dilution of 1:4 (see Fig. 6) and had been shown not to absorb complement and not to be unduly turbid. The complement-fixation titres obtained by the Takatsy manual method are indicated.



is not maximal when the test serum is sampled for only 20 sec. Consequently, a peak on the tracing to be regarded as positive need only be a few optical density divisions (e.g. 0.03 divisions) provided the test sera does not give a similar peak with the liver extract or in the absence of antigen. Test sera giving an organ-specific reaction in a dilution of 1:4 can be titrated against the appropriate antigen as shown in Fig. 7.

#### *Analysis of procedure*

The various stages of the automated procedure were analysed. It is possible to reduce the amount of reagents used below those indicated in Fig. 2, but when this is done there is a tendency for adjacent peaks to overlap. It is likely that the internal diameter of the transmission tubing throughout the system would require to be narrower if smaller volumes of reagents are to be used.

A prolonged period of washing between each sample is required when the reagents have such a high protein content. A sample to wash ratio of 1:5 prevents any overlap between adjacent positive peaks except when the test sera or test antigen is present in high titre.

Various methods of joining the reagent lines after they have passed through the proportioning pump were studied. The most satisfactory results were obtained when capillary tubes were used for the addition of reagents to the main stream. The capillary tubes carrying reagents should lie so that bubbles of air are not trapped at their junctions with the main stream.

The most suitable incubation temperature and time for the reaction between antigen, antibody and complement was studied. It was found that the reaction was not closely dependent on temperature; it would occur at room temperature (20°C) about as efficiently as at 37.5°C. With prolongation of incubation more complement is fixed but there is a tendency for adjacent samples to contaminate each other and for complement to decay. It is therefore necessary to achieve a compromise between these three factors. Because the reservoirs of complement and of antigen require to be kept in an ice bath it was decided that a period of incubation at 37.5°C should be included.

Only a single mixing coil is necessary to achieve an even suspension of the sensitized red cells. The period of incubation for the lysis of the red cells was studied. At this stage the decay of complement is no longer important, but the contamination of adjacent samples with increased incubation is a complicating factor with prolonged incubation. Once again a compromise is achieved between allowing the reaction to go to completion and avoiding diffusion of reagents.

It is estimated that in the flow design as shown in Fig. 2 the fixation of complement and the lysis of the sheep red cells achieve approximately 80% completion.

#### *Manual Methods for Detecting Thyroid and Gastric Complement-Fixing Antibody*

In the indirect fluorescent antibody technique fresh unfixed air-dried sections of snap frozen human gastric mucosa from the body of the stomach were used (Irvine, 1963). The test serum (undiluted) was applied, the sections washed in buffer and then treated with horse anti-human globulin conjugated with fluorescein iso-thiocyanate (Progressive

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Laboratories Ltd, Baltimore; Lot 3122) and then washed in phosphate buffer at pH 7.0. The duration of each stage was 20 min and the whole procedure was done at room temperature. The fluorescein-protein ratio of the conjugate was 8.8  $\mu\text{g}/\text{mg}$ . The conjugate was absorbed with liver and dialysed free of fluorescein. A cover slip was mounted using 10% glycerine in buffer and the sections were examined under ultraviolet light using a Zeiss standard Universal microscope with 200 HBO mercury vapour lamp, dark ground condenser and filters BG 12 and 53. The intensity of staining of the parietal cells was graded as ++, or +, or negative, subjectively.

In the manual complement-fixation method the complement was titred in test tubes. A series of ten dilutions of complement was made in complement-fixation test diluent at 1:10, 1:20, 1:30, . . . 1:100. Two rows of test tubes were then set out. One volume (0.2 ml) of the corresponding dilution of complement was placed in each of the tubes according to the tube number from 1 to 10. Two volumes of complement-fixation test diluent were added using an automatic pipette to each of the tubes in row I and 1 volume of the diluent was added to each of the tubes in row II. One volume of the antigen in the dilution to be subsequently used in the screening of test sera was placed in each of the tubes in row II. One volume of 2% sheep red cells sensitized with haemolysin was then added to all tubes in rows I and II. The tubes were then mixed and placed in a 37°C water bath for 30 min. The complement titre was taken as the tube showing 100% lysis at this time.

The sera were screened and titred using the microtitre equipment supplied by Cooke Engineering Co., 735 North St Asaph Street, Alexandria, Virginia, U.S.A. All sera prior to testing were inactivated at 56°C for 30 min. In two rows of the microtitre trays duplicate doubling dilutions from 1:2 to 1:1024 were prepared for each serum using capillary loops (0.025 ml capacity). A control was added to the end of the first row and this contained saline in place of serum. An equal volume (0.025 ml) of antigen at the appropriate dilution (*vide infra*) was added to all the wells of the first row using the microtitre dropping pipette (1 drop). An equal volume of saline was added to all wells in the second row using a similar dropping pipette. Then to all wells in both rows was added 0.025 ml complement solution containing 2.0 minimum haemolytic doses per ml. After shaking, the plates were incubated at 37°C for 1½ hr. Then to each well was added 0.025 ml 2% sensitized sheep red cells, the trays shaken and incubated for a further 45 min. The trays were then removed, gently shaken once more and replaced in the incubator for a further 30 min. The trays were then left at 4°C overnight and read next morning when they had reached room temperature. The end-point was taken as 50% haemolysis and was read by eye.

To determine the antigenicity of a tissue extract a chessboard titration was done in the microtitre plates using the same procedure as above but with dilutions of a known anti-serum titrated against dilutions of the antigen.

### ANALYSIS OF RESULTS WITH THE AUTO-ANALYZER

The findings obtained with the Auto-Analyzer using the flow design as shown in Fig. 2 were compared with the results obtained with the Takatsy microtitre complement-fixation method and the fluorescent antibody technique for gastric parietal cell antibody (Table 1) and for thyroid microsomal antibody (Table 2). The height of the peak of complement fixation shown by the Auto-Analyzer when the test sera were used in a dilution of 1:4

TABLE 1. A comparison of the methods for detecting parietal cell antibody

Indirect fluorescent antibody method (undiluted serum)	Complement fixation		No. of sera
	Manual (titre)	Auto-Analyzer (sera at 1:4 dilution) (height of peak)	
++	$\geq 32$	+++	27
		++	10
		+	2
		Negative	—
++	$\geq 4 \leq 16$	+++	—
		++	2
		+	9
		Negative	1
+	$\geq 4 \leq 16$	+++	—
		++	1
		+	8
		Negative	6
+	< 4	+++	—
		++	—
		+	3
		Negative	8
Negative	< 4	+++	—
		++	—
		+	—
		Negative	155

correlated well with the titre of the gastric complement-fixing antibody indicated by the manual method and the intensity of staining in the indirect fluorescent antibody technique. The sensitivity of the Auto-Analyzer method is similar to that of the manual technique but slightly less than that of the indirect fluorescent antibody technique. It is to be noted that undiluted serum was used in the fluorescent antibody technique. No false positive results were obtained out of the 232 sera tested for gastric complement-fixing antibody. With regard to thyroid complement-fixing antibody, a similar correlation was found between the results of the manual, immunofluorescence and automated technique using the same sera (Table 2). The titres of complement-fixing thyroid antibody as determined in fourteen sera by the automated and by the manual methods are compared in Fig. 8. Provided the criteria for the selection of the complement titre in the automated method was strictly adhered to, the reproducibility of the titre for complement-fixing antibody as determined by the Auto-Analyzer was extremely precise on account of the graded height of the peaks as the antibody was titred out. The height of the individual peaks on repeated titration was also highly reproducible. By comparison the reproducibility in the manual method was poor at  $\pm 1$  doubling dilution.

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TABLE 2. A comparison of the methods for detecting thyroid microsomal antibody

Indirect fluorescent antibody method (undiluted serum)	Complement fixation		No. of sera
	Manual (titre)	Auto-Analyzer (sera at 1:4 dilution) (height of peak)	
++	$\geq 32$	+++	34
		++	7
		+	3
		Negative	—
++	$\geq 4 \leq 16$	+++	2
		++	3
		+	5
		Negative	2
+	$\geq 4 \leq 16$	+++	—
		++	2
		+	4
		Negative	5
+	< 4	+++	—
		++	—
		+	6
		Negative	21
Negative	< 4	+++	—
		++	—
		+	—
		Negative	138

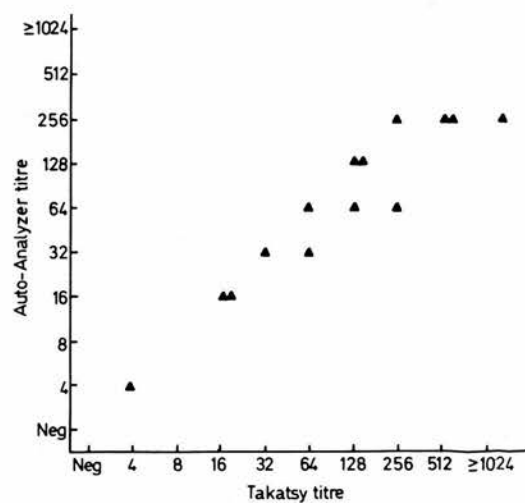


FIG. 8. Correlation between the titres of complement-fixing antibody as determined in fourteen sera by the Auto-Analyzer and the Takatsy manual method.



## DISCUSSION

An automated technique for the detection of complement-fixing antibody to thyroid and to gastric mucosa has been established. The method has the advantage over the manual complement-fixation test of greater precision and reproducibility; a larger number of sera can be tested in a given period and the technique is not so demanding on technician skill. The subjective element in the interpretation of results has been removed. In an ordinary day's work, forty sera can be screened against thyroid, stomach, liver and in the absence of antigen. A disadvantage of the method as presently designed is the volume of antigen used. So far only human thyrotoxic tissue and human partial gastrectomy specimens have been studied. In large hospitals a sufficient number of thyroidectomies are likely to be done to ensure an adequate supply of thyroid tissue but this may not be the case with regard to gastrectomies. The species specificity of parietal cell antibody is wide and it is likely that the gastric mucosa from other species may prove to be suitable. Preliminary studies have suggested that pig gastric mucosa, but not dog, may be used as a source of parietal cell antigen. Further studies are required to determine the most suitable method of preparing the tissue antigens for use in the Auto-Analyzer in the hope that the period of washing between individual test sera may be reduced. This would accelerate the rate of sampling and reduce the consumption of antigen per serum tested.

The Auto-Analyzer method should have general application to the clinical laboratory for the routine screening and titration of sera for gastric and thyroid complement-fixing antibody. It also has application to the research laboratory on account of the greater accuracy and reproducibility in the titration of sera and antigens.

## ACKNOWLEDGMENTS

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The application of microphotometry to the indirect immuno-  
fluorescent antibody technique using the human gastric parietal cell  
antigen-antibody system

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### TECHNIQUES

## THE APPLICATION OF MICROPHOTOMETRY TO THE INDIRECT IMMUNOFLUORESCENT ANTIBODY TECHNIQUE USING THE HUMAN GASTRIC PARIETAL CELL ANTIGEN-ANTIBODY SYSTEM

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### SUMMARY

This paper is an account of our experience in the use of the Leitz microphotometer equipment in the attempt to quantitate the degree of specific fluorescence in the indirect immunofluorescent antibody technique using the human gastric parietal cell antigen-antibody system. The main limitation of the equipment at present is the variability of the HBP 200 W lamp. This fact, together with the biological variation within the tissue section, makes multiple readings necessary. The method is, therefore, time consuming and perhaps mainly applicable to the investigation of standardization of the immunofluorescent technique itself and of the reagents used rather than the quantitation of results obtained in a routine manner using an accepted technique.

### INTRODUCTION

Standardization in the immunofluorescent antibody technique can only be achieved if quantitation is used in each stage of the procedure, including microscopy. Microphotometry has been studied by a number of investigators (Mansberg & Kusnetz, 1966; Pittman, *et al.*, 1967; Goldman, 1967) but so far no instrument has been described that is suitable for the ordinary immunology laboratory. A few of the largest manufacturers are now making microphotometry equipment available. The following is an account of our experience in

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attempting to standardize the indirect immunofluorescent antibody technique using microphotometry applied to the parietal cell antigen-antibody system in man with equipment provided by Leitz, Wetzlar, Germany.

## MATERIALS AND METHODS

### *Instrumentation*

#### *Microscope*

A Leitz Orthoplan large research microscope with a binocular head, phototube and beam-splitter was used. For the present measurements, a fluorite system oil immersion objective FI 95/1.32-1.10 with iris diaphragm was used in conjunction with a periplan GF  $\times 10$  eyepiece in the phototube and dark field oil condenser N.A. 1.20. The viewing eyepieces in the binocular head were periplan GF  $\times 12$ .

#### *Light source and filters*

The light source was a high pressure mercury vapour lamp Osram HBO 200 W fitted in Leitz lamp housing 250 with a reflector and a glass collector. The controls for positioning the lamp, reflector and condenser are placed outside the housing so as to provide rapid and precise centring of the illuminating beam. In addition to a built-in heat absorption filter KG1 2-mm, the lamp housing also included a filter changer which accommodated four transmission filters. These can be inserted in the beam in any desired order by external levers. A neutral diffusing filter together with blue BG12 filters of 5 and/or 3 mm thickness were used for fluorescence excitation. Secondary filter K150 was used for suppression. The microscope was also fitted with a tungsten light source. The object could be examined either under ultraviolet-blue or tungsten light.

#### *Photometer*

Microscope photometer MPV with the Knott light measuring device type MFLK, BN 5001 T was used. The light meter consisted of the photomultiplier measuring head type SKV 317 V, photomultiplier type 6094A (S 11 cathode) and the highly stabilized power supply 500-2500 V, 5 mA type NSHM. As shown in Fig. 1, the MPV photometer attachment consisted of a basic body fitted with a viewing telescope, a variable measuring diaphragm (which may be circular or rectangular), housing for a pilot lamp (8 V, 0.3 A) to illuminate the image of the measuring diaphragm, a dividing plate operated by a lever to expose the photomultiplier attachment to light from the object or to enable the image of the measuring diaphragm to be illuminated by the pilot lamp.

The eyepiece (GF  $\times 12$ ) of the viewing telescope had a graticule for reading the aperture of the measuring diaphragm. The aperture of the diaphragm can be adjusted to any position throughout a continuous range from full field down to an area of approximately  $0.5 \mu^2$  or  $0.5 \mu$  in diameter.

The Scalamp Galvanometer (Pye, Cambridge, England) served as a measuring instrument. It provided four ranges of sensitivity; full, 1/20, 1/100, 1/1000.

The adjustment of the condenser and objective was carried out in accordance with standard methods of microscopy using tungsten light. The centring of the mercury vapour lamp was checked. The condenser, object slides and coverglasses were thoroughly cleaned. The thick-



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ness of the object slides should be between 0.9 and 1.1 mm. For the sake of uniformity, slides of 1.05 mm thickness measured by micrometer were used throughout the present experiment. Coverglass of thickness as near to 0.17 as possible were chosen. Photometer measurements were made in the evenings when powerful electrical equipment in the vicinity was not in use, thus avoiding major fluctuations in the voltage supply which might occur in spite of stabilization.

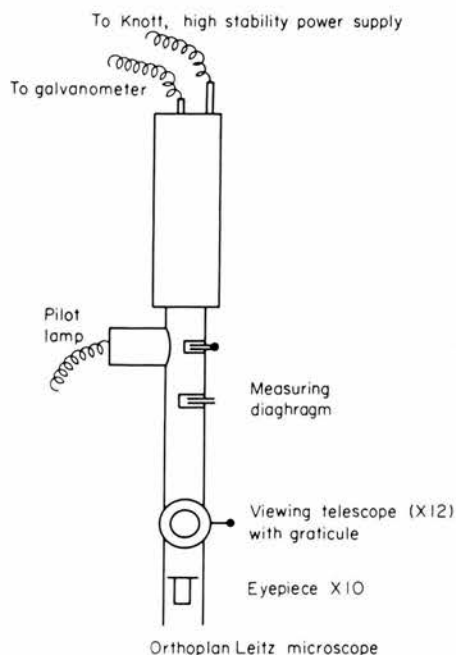


FIG. 1. Diagram of microphotometer attachment.

#### *Fluorescent antibody technique*

The indirect fluorescent antibody technique (Coons & Kaplan, 1950) was used.

#### *Sera*

*Serum B2214* was strongly positive for parietal cell antibody and was obtained from a patient with pernicious anaemia.

*Serum B8805* was negative for gastric parietal cell antibody and was obtained from a control subject. The sera were stored at  $-20^{\circ}\text{C}$ . Freezing and thawing were carried out as rapidly as possible.

#### *Tissue*

Mucosa from the body of human stomach was obtained from a patient undergoing gastrectomy. The fresh tissue was cut into rectangular blocks of approximately 2–3 mm thickness, placed in small glass vials and rapidly frozen to  $-70^{\circ}\text{C}$  in acetone–solid  $\text{CO}_2$  mixture. The blocks were stored at  $-20^{\circ}\text{C}$  until required for sectioning. Sections were cut as uniformly as possible at  $4\mu$  using a Pearce cryostat at  $-20^{\circ}\text{C}$ , air dried by fan for 4 hr,

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stored at 4°C and used within 3 days. During storage, the sections were protected in polythene bags from condensation and prior to use the sections were allowed to come to room temperature before being removed from the polythene bag.

#### *Conjugate*

Fluorescein in the form of fluorescein isothiocyanate (chromatographically pure isomer I) was conjugated with monovalent antiserum—anti-human IgG in our own laboratory. The horse anti-human IgG was obtained from the Central Blood Transfusion Laboratory, Netherlands Red Cross, Amsterdam. For the purposes of conjugation, the globulin fraction was diluted to approximately 10 mg/ml with carbonate–bicarbonate buffered saline at pH 9. Chromatographically pure isomer I fluorescein isothiocyanate (FITC) was used at a concentration of 1 mg/100 mg protein. The conjugation was done at 4°C for 18 hr. Free fluorescein was removed by filtration through Sephadex G-50 followed by overnight dialysis in veronal buffer, pH 7.2 (Oxoid, England) at 4°C. The fluorescein–protein absorption ratio was 0.67 as determined by Zeiss PMQ II and M4Q III spectrophotometer equipment using wave band 495 mμ for fluorescein and 280 mμ for protein.

The same batch of conjugate (GG4 Lot 11) was used throughout the studies described in this paper.

#### *Immunofluorescent staining*

Undiluted and diluted serum, after warming to room temperature, was applied to each section for 20 min at room temperature. The sections were then washed in veronal buffer (pH 7.2) for 30 min with continual gentle agitation on a rolamix machine (Luckham, England), the buffer being changed once during this procedure. The fluorescein–protein conjugate (anti-human IgG–FITC) was applied for 20 min at room temperature. The sections were then given a final wash in veronal buffer for 1 hr with continuous agitation and changing of the buffer every 20 min. The sections were then mounted in 10% glycerol in veronal buffer.

#### *Fluorescent standard*

A small quantity of Zn–Cd sulphide fluor, lot 1023, was obtained through the kindness of Dr Morris Goldman, Department of Immunology, Bionetics Research Laboratories Inc., Falls Church, Virginia. An amount smaller than visible by the naked eye was placed on a clean glass slide. The preparation was then mounted with a coverslip using non-aqueous univert mountant (Gurr, England). After allowing the mounting medium to set firmly, a single crystal was selected as fluorescent standard using the same objective (×95), condenser and other optics as above. The iris of the objective was always set in the closed position.

## RESULTS

#### *Standardization of microscope*

The intensity of a new lamp HBO 200 W falls off rapidly within the first few hours of its life and then enters a plateau phase. Using the fluorescent standard, as described above, it was apparent that the intensity of the beam from the HBO 200 W lamp in its plateau phase may remain constant over a period of several hours but that it may suddenly fluctuate by up to 15–20% due to instability of the arc of the lamp.

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### *Assessment of optimal dilution of conjugate*

Sections of gastric mucosa were stained in the indirect immunofluorescent technique using positive antiserum B2214 diluted 1:2 in veronal buffer and using serial dilutions of anti-human IgG-FITC conjugate (1:2, 1:4, 1:6, 1:8, 1:10, 1:16 and 1:20). The fluorescence of the cytoplasm was measured in fifty parietal cells in each slide, there being one slide for this purpose for each dilution of the conjugate. The cells were chosen at random throughout the section provided they had an area of cytoplasm large enough to accommodate the square area determined by the measuring diaphragm ( $10 \times 10$  on the eyepiece graticule

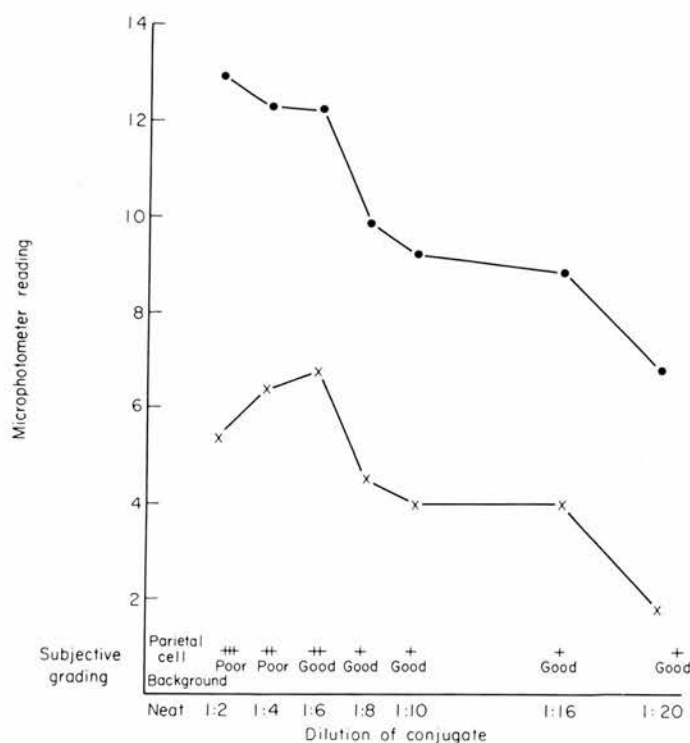


FIG. 2. Determination of optimum dilution of conjugate for use in the indirect immunofluorescent antibody technique. The photometer readings for the contrast between specific (●) and non-specific (×) fluorescence correlate well with the subjective assessment, indicating that a dilution of conjugate at 1:6 is optimum. Positive serum B2214 was used in a dilution of 1:2 throughout. Primary filter 5 mm BG12. The results are shown as the average of measurements on fifty cells for each dilution of conjugate.

of the viewing telescope). Care was taken to ensure sharp focus on the parietal cell cytoplasm and that the area to be measured did not overlap onto the nucleus. This reading was designated as 'specific fluorescence'. The reading from an area of the same size adjacent to each cell was measured in each instance and designated the 'non-specific fluorescence'. The difference between the 'specific' and the 'non-specific' fluorescence is referred to as 'the contrast'. The primary filter in this experiment was Schott 5 mm BG12. The microphotometer readings

were made as quickly as possible (2–3 sec) in order to avoid undue exposure of the tissue section to ultraviolet–blue light. The light beam was switched to tungsten light whenever measurements were not being taken.

Duplicate slides were read subjectively for brightness of parietal cell fluorescence (specific fluorescence) and for brightness of background tissue staining (non-specific fluorescence). The results were correlated with the mean value of the photometer readings for each slide and are shown in Fig. 2. It is seen that the optimum dilution of conjugate to give maximum contrast between specific and non-specific fluorescence was found to be 1:6 both by micro-photometry and by subjective assessment.

TABLE 1. Average of micrometer readings of fifty cells in each of ten slides stained with a positive serum and on each of eight slides stained with a negative serum

		Average of fifty readings		
		Specific	Non-specific	Contrast
Undiluted +ve serum B2214				
Slide	1	15	7.15	7.85
	2	14	6.20	7.80
	3	14.34	6.78	7.56
	4	11.74	5.95	5.79
	5	11.67	5.78	5.89
	6	13.00	6.54	6.46
	7	12.10	6.10	6.00
	8	12.94	6.50	6.44
	9	11.55	5.60	5.95
	10	11.47	5.25	6.22
Undiluted –ve serum B8805				
Slide	1	8.55	7.68	0.87
	2	7.67	6.78	0.89
	3	6.18	5.45	0.73
	4	6.48	5.84	0.64
	5	6.73	5.94	0.79
	6	7.16	6.50	0.66
	7	8.88	5.21	0.67
	8	5.96	5.23	0.73

*Reproducibility of readings from gastric parietal cells showing positive immunofluorescence*

Ten slides of gastric sections stained with undiluted positive serum B2214 and eight slides stained with undiluted negative serum B8805 were studied. The conjugate was used in a dilution of 1:6. Measurements were carried out on different days but always within 2 hr of staining.

As in the above experiment, the fluorescence of the cytoplasm was measured in fifty parietal cells in each slide. The measurements were conducted in the same way as above. The findings are shown in Table 1. The standard deviation for the contrast between specific and non-specific fluorescence for the fifty parietal cells measured in slide 10, as an example,



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was  $\pm 1.104$  with a mean of 6.22 and a range of 4.8–9.1. There was no consistent decline in the immunofluorescent readings to indicate that loss of fluorescence had occurred because of exposure to ultraviolet-blue light of those parietal cells that were read towards the end of the series in each slide using intermittent exposure.

*Titre of antibody determined by microphotometry and by subjective assessment*

Serial doubling dilutions of positive serum B2214 were tested against duplicate slides and microphotometry carried out as described above, the fluorescence of the cytoplasm of fifty

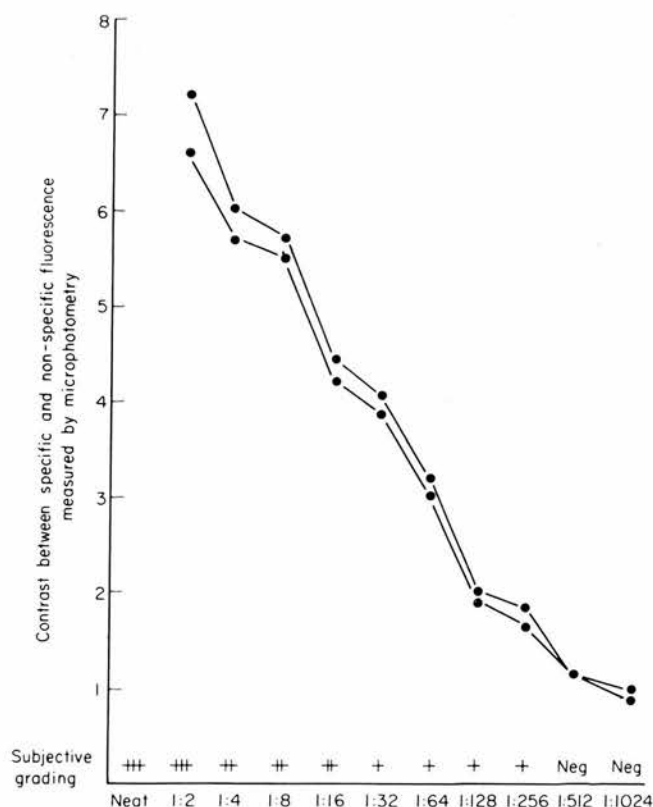


FIG. 3. The correlation between the microphotometry readings and the subjective assessment of positivity with serial dilutions of positive serum B2214. Measurements were made on duplicate slides for each dilution of the serum. The results are expressed as the mean of the measurements on fifty parietal cells on each slide. Primary filter 5 mm BG12.

parietal cells compared to that of adjacent tissue being measured in each slide. The results of photometry were correlated with the subjective grading of positivity in each of the slides. The anti-human IgG-FITC conjugate was used in a dilution of 1:6. Duplicate slides were always examined with the same microphotometer settings and on the same day (1.4 kV, sensitivity 1/20, primary filter 5 mm BG12). The results are shown in Fig. 3.

*Photodecomposition of fluorescein conjugate on exposure to ultraviolet-blue light*

Fig. 4 shows the fall-off in intensity of fluorescence in the gastric parietal cell antigen-antibody system on continuous exposure of individual cells to ultraviolet-blue light using primary filter 5 mm BG12 and primary filter 5+3 mm BG12. It is clear that the fall-off in immunofluorescence is more rapid with the 5 mm BG12 filter as compared to the 5+3 mm BG12 filter although the intensity of fluorescence is much higher, especially initially with only the 5-mm filter. However, a greater contrast between the specific staining and the non-specific staining is obtained for a longer period with the 5+3 mm BG12 filters.

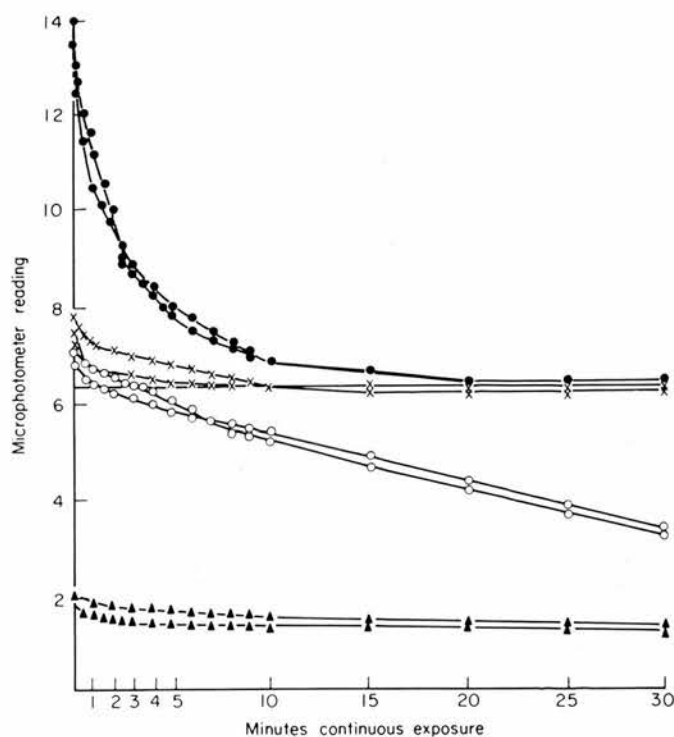


FIG. 4. The microphotometer readings of the cytoplasm of gastric parietal cells stained in the indirect immunofluorescent antibody technique (specific fluorescence) and of the adjacent gastric tissue (non-specific fluorescence). The readings were taken during continuous exposure to ultraviolet-blue light using primary filters 5 mm BG12 and 5+3 mm BG12, respectively. ●, Specific (primary filter 5 mm BG12); ×, non-specific (primary filter 5 mm BG12); ○, specific (primary filter 5+3 mm BG12); ▲, non-specific (primary filter 5+3 mm BG12).

At this juncture, a new mercury HBO 200 W lamp was introduced giving a much greater intensity of illumination than the previous lamp that had been used for many hours. The setting of the photometer had to be altered entirely with regard to voltage sensitivity. Fig. 5 shows the effect of continuous exposure on specific staining to excitation using a 5+3 mm BG12 blue primary filter compared to the effect of intermittent exposure when the beam

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was switched to tungsten light between the readings, each of which lasted from 2 to 3 sec. Fig. 5 shows that the greater the initial intensity of the fluorescence the more rapid is the photodecomposition, as illustrated by curves 1-4 which are consecutive during the early hours of the lamp's life. The greater intensity of illumination produced by excitation with 5 mm BG12 filter caused more rapid photodecomposition of fluorescence than a weaker intensity of illumination produced by 5+3 mm BG12. Intermittent exposure by radiation produced by the combination of 5+3 mm BG12 gave very little fading of fluorescence and this filter arrangement was, therefore, used for the following experiment.

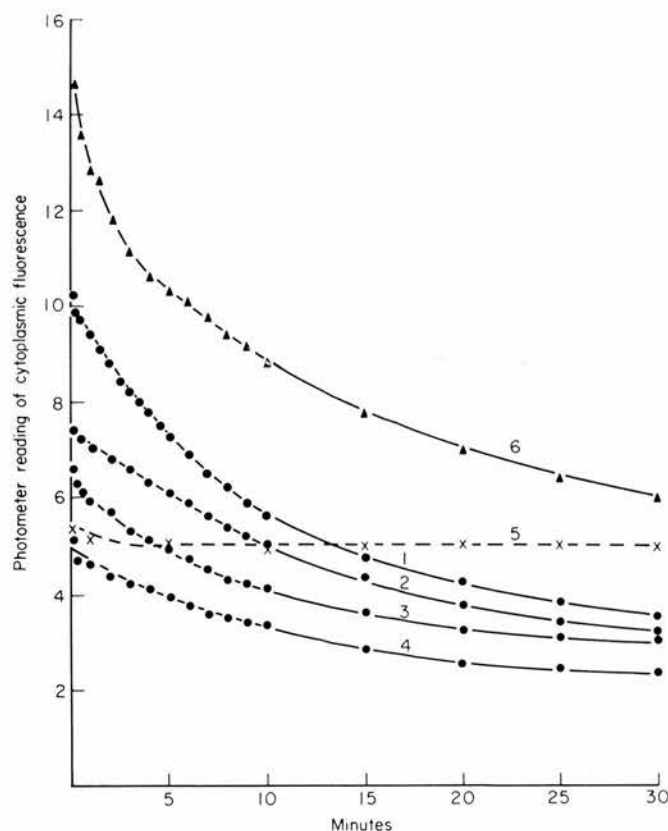


FIG. 5. Photometer readings from the cytoplasm of gastric parietal cells stained in the indirect immunofluorescent antibody technique with serum B2214. Curves 1-6 are consecutive. The cells were subjected to continuous exposure to ultraviolet-blue light except in experiment 5, when the exposure was intermittent (approximately 2 sec for each measurement). Primary filters as shown. ▲, 5 mm BG12, continuous exposure; ×, 5+3 mm BG12, intermittent exposure; ●, 5+3 mm BG12, continuous exposure.

*Comparison of intensity of immunofluorescence of gastric parietal cells in the upper and lower part of the gastric crypts*

Four sections of gastric mucosa were stained with neat positive serum B2214 and the anti-IgG-FITC conjugate in a dilution of 1:6. Forty parietal cells were measured for each

slide, twenty chosen from cells in the region of the mucous neck glands and twenty chosen from deep in the gastric crypts. The cells were selected from the superficial and deep parts of the gastric sections alternately using tungsten light. The exposure of the gastric sections to ultraviolet-blue illumination was intermittent and for the measurement of fluorescence only. Primary filters 5+3 mm BG12 were used. The aperture of the measuring diaphragm was  $10 \times 10$  as throughout the whole of the series of experiments. The results of this study are shown in Fig. 6. It is clear that the intensity of fluorescence of the deeper cells is statistically greater than is the immunofluorescence intensity of the more superficial gastric parietal cells. This difference is apparent subjectively (see Fig. 1 of Irvine, 1963).

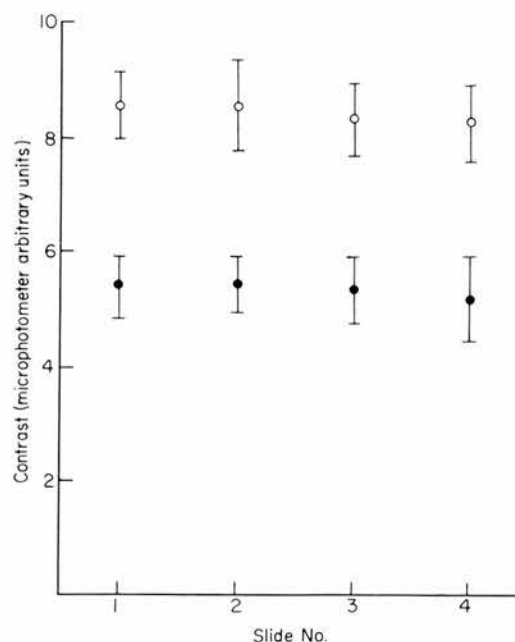


FIG. 6. Illustrates the difference in the intensity in positive immunofluorescence (specific minus non-specific) in the cytoplasm of human gastric parietal cells in the upper (●) and in the deeper (○) part of the crypts. Four sections were stained by the indirect immunofluorescent method with serum B2214. Twenty superficial and twenty cells deep in the gastric mucosa were measured with the microphotometer. The results are shown as the mean values  $\pm 1$  SD.

## DISCUSSION

One inherent defect in the present system is the instability of the arc of the HBO 200 W lamp. This may be overcome by substituting an HBO 100 lamp. The stability of the arc of this latter lamp would merit the provision of a highly stabilized voltage unit for that lamp.

The variation in stability of the HBO 200 W lamps in their plateau phase was estimated to be 15–20% in the present study and the oscillation within that range was random. For periods of variable length, the lamp could be quite stable ( $\pm 1\%$ ) and then oscillate. The fluctuation in the intensity of the light source would, therefore, be compensated for to some



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extent by taking multiple readings and determining the mean value with standard deviations where necessary.

Biological as well as instrumental variability must also affect the degree of reproducibility of the photometer readings. Even if the perfect tissue section could be cut so as to be of uniform thickness throughout, the cytoplasm of different parietal cells would be cut at varying angles so that different thicknesses of fluorescing material may be available for different cells. As shown in Fig. 6 of the present paper, parietal cells situated in different parts of the gastric section vary in their intensity of fluorescence in the fluorescent antibody technique. Cryostat sections are not perfect sections. They are likely to vary in average thickness and in thickness within each section. This must affect the amount of fluorescence emitted, particularly when transmitted rather than incident light is used. Again, as with the light source, the variation in these factors would be random or can be randomized. Hence, remarkably reproducible results may be achieved (see Figs. 2 and 4) when the mean readings from fifty cells are plotted for duplicate slides with serial dilutions of antiserum.

In spite of the extreme sensitivity of the microphotometer, it is of interest that, at least with regard to the gastric parietal cell antigen-antibody system, the findings with microphotometry correlate very closely with the subjective interpretation. This was particularly clear in the titration of an antiserum.

The main application of microphotometry in immunofluorescence work at the present time would appear to be in defining the procedure of preparing immunofluorescence specimens to give optimum results. Microphotometry should be useful in determining the best buffer to use as diluent, the optimum dilution of conjugate and its staining characteristics (Pittman *et al.*, 1967) in comparison to a standard conjugate, washing times for sections to give optimal contrast between specific and non-specific fluorescence, the most suitable mounting medium for fluorescence preparations, the most appropriate primary and secondary filters and the choice of other optics to give maximum contrast and minimum background fluorescence and as a check on the performance of the lamp.

#### ACKNOWLEDGMENT

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